Developmental Origins of Aggressive Medulloblastoma

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Abstract

Medulloblastomas represent a heterogeneous group of cerebellar tumors that constitute the most frequent primary pediatric solid malignancy. Molecular characterization of these tumors have led to the understanding that distinct subtypes possess characteristic properties such as gene expression profile, histological classification, and degree of dissemination that are predictive of disease progression and prognosis. Fractionation of primary medulloblastomas has led to the appreciation of brain tumor stem cells (BTSC) that may be driving the more aggressive and malignant disease. However, the developmental origins of these cells as well as the influences of early mutations in tumor suppressors on development and tumorigenesis remain unclear.

My work is geared towards understanding the impact of mutations in the key tumor suppressor genes Ptc1 and p53 on medulloblastoma formation. I first identified key differences in neural stem cell marker expression that distinguish between Ptc1 and Ptc1;p53 medulloblastomas, demonstrating that the Ptc1;p53 genotype may pre-dispose to a more malignant, stem-like tumor. Through the use of a somatic mosaic model, we describe a synergistic interaction between Ptc1 haploinsufficiency and p53 deficiency leading to developmental seeding of the cerebellar field by pre-malignant cells and term this phenomenon “developmental field cancerization.” Interestingly, we observed this pre-malignant colonization in the cerebellar stem cell compartment as well, resulting in an

aberrant population of self-renewing cells. Upon loss-of-heterozygosity at the Ptc1 locus, the Ptc;p53 animals alone develop robust cerebellar tumors that possess a definable stem-like population of cells that can re-initiate metastatic secondary tumors. These findings demonstrate how early mutations in the tumor suppressor genes, such as Ptc1 and p53, may lead to stem cell field cancerization and play an important role in determining future tumor character and prognosis.

Finally, bisulfite-based mass spectrometry methylation assays were utilized to define differences in methylation status at various imprinted loci between BTSC and bulk tumor. We observed methylation differences at the KCNQ1OT1/Lit1 and CDKN1C/p57 and corroborated these findings with mRNA expression and RNA-FISH demonstrating bi-allelic expression of Lit1 in the BTSC. Through lentiviral-mediated shRNA knockdown of Lit1 in BTSC, I ascribe a putative role for the long non-coding RNA in maintenance of BTSC self-renewal.

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List of Abbreviations

Adenomatous polyposis coli	APC
Basic Helix-Loop-Helix	bHLH
Beta-catenin	Ctnnb1
Brain Tumor Stem Cell	BTSC
Cerebellar Stem Cell	CbSC
Cerebellum	Cb
Central Nervous System	CNS
Cyclin-dependent kinase inhibitor 1C	Cdkn1c
DNA methyltransferase	Dnmt
Embryonic Stem Cell	ES
Epidermal Growth Factor	EGF
External Granule Layer	EGL
Fibroblast Growth Factor	FGF
Granule Cells	GC
Granule Cell Progenitor	GCP
Green Fluorescent Protein	GFP
Hairy and enhancer of split1	Hes1
Inhibitor of DNA binding 2	Id2
Internal Granule Layer	IGL
KCNQ1 overlapping transcript 1	Kcnq1ot1
Loss-of-Heterozygosity	LOH
Loss-of-Imprinting	LOI
Magnetic Resonance Imaging	MRI
Mouse Atonal Homolog 1	Math1
Neurosphere Media	NS-M
Patched-1	Ptc1
Purkinje Cell	PC
Short hairpin RNA	shRNA
Smoothened	Smo
Sonic Hedgehog	Shh
SRY (sex determining region)-box 2	Sox2
Suppressor of Fused	Sufu
T-Acute Lymphoblastic Leukemia	T-ALL
Tissue Stem Cell	TiSC
Trichostatin A	TSA
Wild-type	WT
Wingless	Wnt
5-Aza-deoxycytidine	5Aza

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Chapter 1

Introduction

Introduction

Medulloblastomas are the most common primary childhood CNS tumor, representing 16% of all pediatric brain tumors with about one thousand new cases every year[1]. The incidence of these cerebellar tumors is bimodal with peaks at 3-4 and 8-9 years of age[2]. Affected patients usually present with headache, vomiting, lethargy, as well as increasing head circumference secondary to increased intracranial pressure and obstructive hydrocephalus. The treatment of these tumors may be complicated by extensive local invasion, making total surgical resection difficult. Furthermore, craniospinal metastases, which occur in 11-43% of patients, are associated with significantly worse prognosis and require radiation and systemic chemotherapy, leading to decreased quality of life (Figure 1-1). While there have been great advances in our understanding of how these tumors arise as well as in development of new treatment protocols, patients with the most aggressive forms of medulloblastoma still have a five-year event free survival of only 30% and frequently suffer from iatrogenic developmental and cognitive deficits. Thus, further elucidation of the factors and pathways driving malignant progression could yield insights leading to the development of more targeted therapies and better risk-stratification.

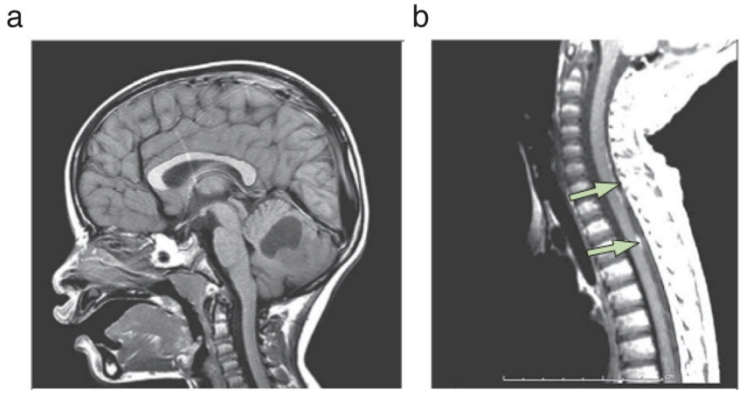

Figure 1-1 | Characteristic MRI features of medulloblastoma
a, Sagittal T1-weighted imaging showing a cerebellar mass, typically found midline. b, Leptomeningeal metastases to the spinal cord is not uncommon (marked). Images obtained from Crawford *et al*[3].

In their classic review, Cushing and Bailey first proposed that embryonal rests in the developing cerebellum give rise to medulloblastomas, citing their early incidence as well as the undifferentiated cells constituting these fast-growing cerebellar tumors[4]. Indeed, as our understanding of their cellular origins has grown, it has become increasingly apparent that medulloblastomas arise from normal development gone awry leading to the uncontrolled expansion of progenitor cells, widely thought to be granule cell progenitors (GCP)[5].Thus, it is important to begin with a basic understanding of the processes driving normal development and differentiation of the cerebellar organ.

Overview of mouse cerebellar development

The cerebellum is involved in facilitating a number of basic cognitive functions, including voluntary motor movement coordination, balance, equilibrium, and muscle tone (it facilitates muscle tone?) as well as motor learning, speech, and spatial memory[5]. Developmentally, the different cell types of the cerebellum (illustrated in Figure 1-2) arise from two distinct embryonic germinal zones[6]. The dorsomedial ventricular zone gives rise to the Purkinje cells as well as many of the cerebellar interneurons: Golgi, basket, and stellate cells. The rhombic lip gives rise to primarily the granule neurons, the most numerous cell type in the cerebellum and, indeed, in the entire brain.

The earliest cerebellar progenitors originate from the ventricular zone at approximately embryonic (E) day E10.25. These cells are characterized by expression of the transcription factors Lhx2/9, Meis 1/2, and Irx3 and give rise to precursor Purkinje neurons by E14, which migrate to seed the developing cerebellar anlage[6]. Interestingly, *Ptf1a* mutations, which prevent generation of Purkinje cells and other GABAergic neurons in the cerebellum, result in complete

cerebellar agenesis[7], underscoring the importance of Purkinje cells and Shh-signaling in establishing the cerebellar field as well as driving its growth.

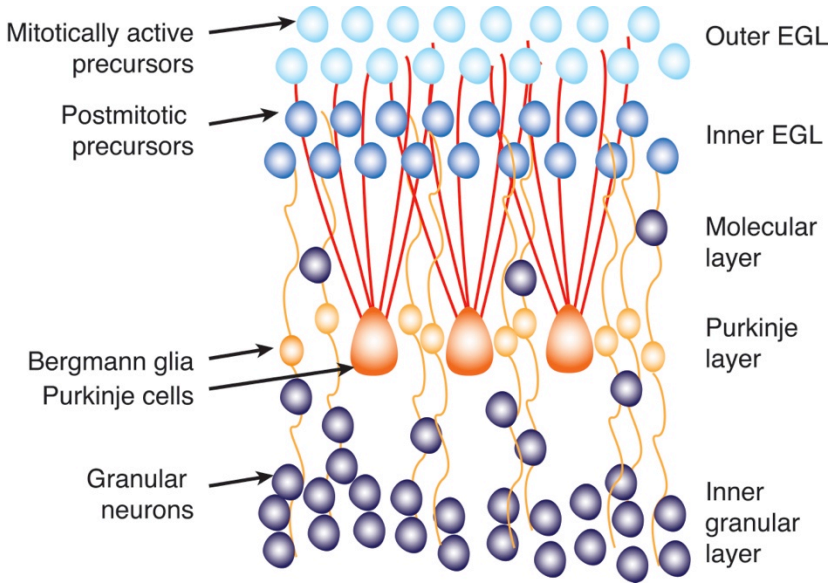

Figure 1-2 | Primary cell types and basic architecture of the developing cerebellum
Schematic representation of the early developing cerebellum adapted from *Ruiz y Altaba* et al[8]. Cell types derived from ventricular zone are shown in orange and red; rhombic lip derivatives are shown in shades of blue and indigo. Sonic Hedgehog (Shh) is secreted by the Purkinje neurons to drive the proliferation of the mitotically active precursor cells in the outer EGL. Post-mitotic precursors migrate inwards along the Bergmann glia and through the Purkinje cell layer to form the internal granule layer. The cell bodies of granule cells reside in the nuclear layer, also known as the internal granule layer (IGL). The Purkinje cells form a separate layer of cells between the IGL from the molecular layer (ML). Golgi cells, also known as Bergmann glia, are interspersed amongst Purkinje cells.

Around E12.5, the rhombic lip gives rise to a second proliferating pool of progenitors characterized by expression of the basic helix-loop-helix (bHLH) transcription factor Atoh1 (Math1), Zic1, as well as Meis1. Math1 is particularly critical for the early specification of these cerebellar progenitor cells[6] and is also involved in cell-fate specification of inner ear hair cells[9] as well as intestinal secretory cells[10]. Within the cerebellum, Math1 is first expressed in the rhombic lip as early as E9.5 and is essential for the development of the cerebellar granule neurons: *Math1*-null animals fail to form granule cells and develop cerebellum lacking an EGL[11].

Regulation of *Math1* takes place at a number of levels and has been shown to occur transcriptionally by Zic1[12], which binds directly to a *Math1* enhancer and represses its transcription, and post-translationally via the Bmp-pathway, which modulates Math1 protein stability[13]. Significantly, deletion of *Math1* in the developing cerebellum disrupts granule cell proliferation and induces differentiation as well as prevents formation of tumors in a Shh-driven mouse medulloblastoma model[14]. Over-expression of Math1 similarly leads to a smaller, less foliated cerebellum likely through pre-mature differentiation of GCPs[15], emphasizing the importance of maintaining appropriate levels of Math1 expression for normal differentiation and production of granule cell progenitors.

These Math1-positive cells subsequently migrate out of the rhombic lip and coat the surface of the developing cerebellar field to form the external granule layer (EGL). At this point, some of the post-mitotic precursors will continue to migrate inwards past the Purkinje cell layer to form some of the cerebellar nuclei, while the vast majority of these rhombic lip derivatives will remain to form a zone of proliferating granule cell progenitors in the outer EGL to give rise to the cerebellar granule cell[16]. This large, clonal expansion begins at approximately postnatal day 2 (P2) and continues until P15, and occurs secondary to secretion of the mitogen Sonic hedgehog (Shh)[17]. The primary source of Shh at this time is the Purkinje neuron, which actively secretes Shh in the developing cerebellum. Binding of Shh to its 12 transmembrane receptor, Ptc1, results in dissociation of Ptc1 from the seven transmembrane G-protein-coupled receptor-like protein Smoothened (Smo) and subsequent de-repression of the Shh signaling pathway (Figure 1-3)[18]. Activation of the Shh signaling pathway leads to activation of the zinc finger proteins Gli1 and Gli2 and inactivation of the transcriptional repressor Gli3, which together go on to drive granule

cell proliferation[17]. By the end of the second postnatal week (P14), granule cell progenitors will finish their proliferative cycle, down-regulate Math1, and exit the cell cycle[13]. As this occurs, post-mitotic granule cells will move inwards from the EGL along the radial fibers of the Bergmann glial cells, pass through the Purkinje cell layer, and form the mature inner granule layer (IGL). In this way, normal cerebellar development is usually considered complete by the third postnatal week (P21).

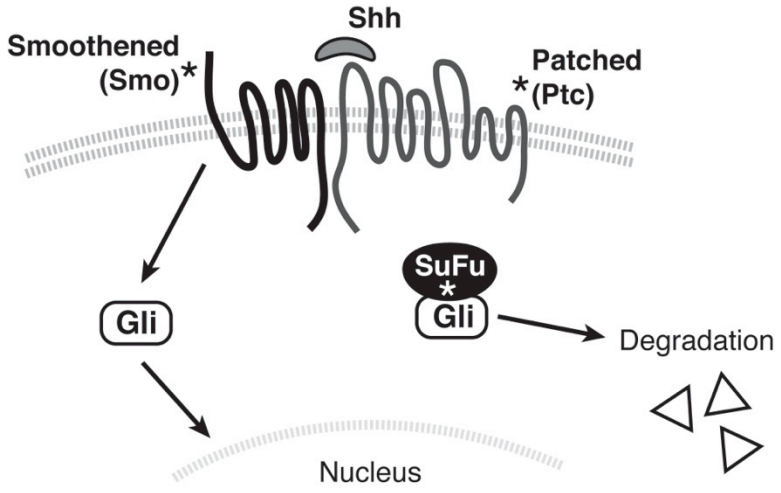

Figure 1-3 | Schematic of the SHH signaling pathway
In the absence of Shh, Ptc binds and prevents Smoothened-mediated activation of the Gli transcription factors. Binding of Shh by Ptc releases Smo, allowing activation of the nuclear translocation of the Glis which activate the downstream effectors of the Shh pathway. Suppressor of Fusion (SUFU) normally binds and facilitates the degradation of Gli. Stars denote proteins found to be mutated in medulloblastomas. Figure adapted from Roussel and Hatten[5].

Underscoring the importance of Shh-signaling in regulating cerebellar development and medulloblastoma tumorigenesis, approximately 30% of human medulloblastomas demonstrate deregulation of the Shh-pathway[2]. Moreover, genetic changes are frequently observed within Shh-pathway genes such as inactivating mutations in Suppressor of fusion (*SUFU)*[19], *Ptc1*[20], activating mutations in *Smo*[21], as well as copy-number gains in *MYCN* and *Gli2*[22]. Additionally, the final granule neuron differentiation and migration steps of cerebellar development are facilitated by expression of the chemokine receptor CXCR4[23] and a number of proteins involved

in glial-guided migration. CXCR4 (CD184) signaling is expressed not only in the immune system, where it is involved in lymphocytic chemotaxis, but also in the central nervous system[24]. The importance of CXCR4 in human tumorigenesis has been substantiated by the appreciation that medulloblastomas expressing high levels of CXCR4 are associated with worse prognosis[25] and small molecule antagonists of CXCR4 inhibit intracranial tumor growth[26]. In mice lacking CXCR4, GCPs have been observed to migrate prematurely along the Bergmann glia and thus decrease the proliferative expansion of these cells in the EGL. Conversely, mouse models that slow or prevent the migration of GCPs along the Bergmann glia through alteration of factors involved in glial cell interaction such as BDNF[27] and Astn1[28] have been shown to lead to ectopic proliferative rests of GCPs that persist in the EGL beyond normal development. These populations of developmentally arrested cells may in fact be similar to those rests found in the cerebellum of Ptc1-mutant mice which frequently develop medulloblastomas[29].

Timing of human cerebellar development

In contrast to the mouse, where the proliferative expansion of the EGL occurs largely postnatally, the human cerebellum undergoes a 5-fold volumetric expansion between weeks 24-40 of gestation. Formation of the EGL, marked by the initiation of cerebellar foliation, occurs between weeks 20-30 and GCP proliferation and migration take place between weeks 30-40. The EGL diminishes following birth throughout the first postnatal year as the IGL continues to increase in volume[30].

Molecular characterization of medulloblastoma

The term medulloblastoma refers to a type of cerebellar tumors that occur primarily during childhood[1]. Over the years since their initial characterization, it has become clear that this term describes a heterogeneous class of tumors that may be characterized by a number of factors that

are associated with variable prognoses: time of incidence, histopathology, and gene expression profiles[31]. Tumors occurring earlier during infancy (<3 years of age) are associated with particularly poor prognoses. Relatively few adults (>16 years of age) develop medulloblastomas, accounting for only 3-4% of primary intracranial malignancies overall.

Histologically, medulloblastomas may be classified into three categories: classic, desmoplastic, and anaplastic (Figure 1-4)[1]. Classic medulloblastomas constitute approximately two thirds of all cases and are characterized by relatively uniform sheets of undifferentiated cells with high nuclei to cytoplasm ratios. Desmoplastic medulloblastomas in contrast have collagen fibers and stroma between tumor cells, and generally have fewer mitotic figures, indicating a less proliferative tumor-type than the classic. Anaplastic tumors are the least common and consist of highly mitotic and undifferentiated cell types that can be particularly resistant to current treatment modalities.

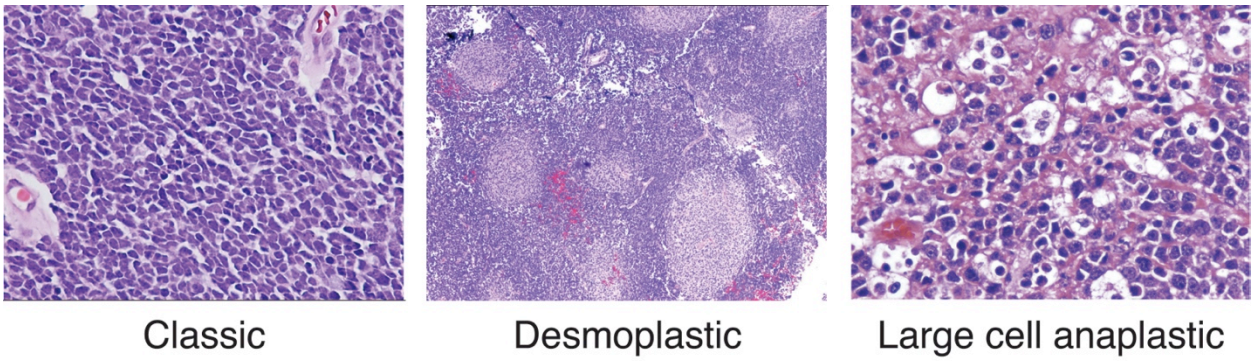

Figure 1-4 | Medulloblastoma can present as different histological subtypes
Representative sections demonstrating microscopic findings for the different histological subtypes of medulloblastoma. Desmoplastic tumors are typically associated with a Shh gene signature and large cell anaplastic tumors are associated with the worst prognoses. Images taken from MedScape.

The greatest leap in our understanding of medulloblastoma biology came with our ability to perform high-throughput genome and expression analyses as a means of profiling the molecular

characteristics of these tumors[31]. These studies were paramount for elucidating key signaling pathways essential for tumor establishment and maintenance as well as for identifying potential driver mutations in tumor formation. As a result, it is now accepted that medulloblastomas may be sub-divided into four groups based on mRNA expression profiles: Wnt, Shh, Group C, and Group D[2]. Mutations in Wnt and Shh pathways usually occur mutually exclusive of one another and lead to tumors with distinct gene expression patterns. Together, Wnt and Shh tumors account for nearly 40% of all medulloblastomas. Group C and D tumors, known also as non-WNT/SHH tumors, are characterized by over-expression of genes involved in neuronal development such as phototransduction and glutamate signaling genes in Group C and semaphorin and beta-adrenergic signaling genes in Group D. Group C tumors are distinct in that they also often exhibit amplification of the oncogene MYC and are most frequently associated with an anaplastic histologic presentation and disseminated disease phenotype, resulting in the worst prognoses of all tumor subgroups. While therapy has remained essentially identical for all subgroups of medulloblastoma until now, the findings from these studies inspired the development of novel mouse models for medulloblastoma that are allowing increased understanding of the differences between medulloblastoma subtypes as well as development of more targeted therapies and treatment protocols that are simultaneously more efficient and less destructive than existing modalities.

Mouse medulloblastoma models for Shh-type tumors

Human medulloblastomas with a Shh gene signature represent the best studied of the tumor subtypes and our understanding of these tumors has benefitted tremendously from the development of murine models for the disease. The initial observation that patients with Gorlin's syndrome, a hereditary autosomal dominant disease associated with inactivating mutations in the

Ptch1 gene[32], are at increased risk for developing a number of malignancies including basal cell carcinoma and medulloblastomas led to the observation of *Ptch1* mutations in sporadic human medulloblastomas[20] as well as the development of one of the first mouse medulloblastoma tumor models [33].

The long latency and low penetrance of medulloblastoma formation in the *Ptch1*-mutant animals[33] implied that additional genetic lesions were required for malignant progression. It was observed that crossing $Ptc1^{+/-}$ mice with *p53*-deficient mice significantly increased medulloblastoma incidence to 95% with increased kinetics as well[34]. The transcription factor Trp53 (p53) has been shown to play a role in suppression of tumorigenesis in a wide range of malignancies. While *p53* gene mutations are only infrequently observed in human medulloblastoma[35,36], it should be noted that their presence is associated with universally poor prognosis[37] and people with Li-Fraumeni syndrome, caused by germ-line mutation of *p53*, are at increased risk for developing medulloblastoma[38]. Additionally, mutations in the protein phosphatase WIP1, a known negative regulator of p53 activity, have been observed in primary medulloblastoma samples as well as cell lines[39]. More recently, amplification and over-expression of the ubiquitination factor *UBE4B* has been observed in human medulloblastoma tumors and shown to negatively regulate the stability and function of p53[40]. Thus, modulation of p53 pathway activity remains an important aspect of medulloblastoma biology to be further studied.

Histological examination of the cerebellum in *Ptc1*-mutant animals identifying ectopic proliferative rests in the EGL first suggested that stalled granule cell progenitors may be the

tumor-initiating cell-of-origin in medulloblastoma[29]. These findings have led to numerous mouse models that focused on genes involved in the Shh-signaling pathway[41] and ultimately to the development of small molecule inhibitors of Smo that were able to suppress and delay tumor formation in transplantation and mouse medulloblastoma models[42,43]. A recent patient case study demonstrated that Smo-antagonists were remarkably effective at alleviating the tumor burden[44,44]. However, in this case, remission was only transiently achieved before the recurrence of insensitive tumor cells[44,45], underscoring the need for the development of better targeted therapeutics as well as for the elucidation of pathways important for maintenance of treatment-resistant medulloblastoma cells.

Brain tumor stem cells

In the course of normal development, pluripotent embryonic stem (ES) cells undergo differentiation into committed cell lineages, giving rise to the different tissues and organs of the body. In doing so, they lose the fundamental characteristics that define them as stem cells: the ability to continually self-renew and give rise to multiple lineages. At the same time, small populations of stem cells continue to persist in the adult that are critical for such functions as tissue maintenance and regeneration. Thus, it is easy to see how homeostatic maintenance of this subpopulation of cells is critical for not only normal development but also the continued maintenance of functionality throughout life. Conversely, transformation of a multi-potent and self-renewing cell could lead to malignancies with increased plasticity, heterogeneity, and resiliency, an idea forming the basis of the cancer stem cell model for tumorigenesis[46].

Cancer stem cells were first identified in leukemia in 1994[47] and have since been observed in a variety of solid tumors. Early transformation of a more stem-like cell could have broad

implications on the characteristics of the subsequent tumor. For example, work within a mouse model for T-ALL utilizing the Sleeping Beauty transposon mutagenesis system has shown that altering the timing of mutagenesis along T-cell development affects the kinetics of disease progression, the number and distribution of driver mutations, as well as the gene expression profiles of the resulting tumors[48]. Furthermore, the conferral of properties affiliated with normal tissue stem cells, such as slower cell cycle rate, enhanced DNA damage repair ability, and the expression of multidrug efflux pumps makes tumors with a defined cancer stem cell compartment more refractive to conventional radiation and chemotherapeutic treatment regimens[49,50]. Thus, the functional characteristics defining a cancer stem cell can be seen as possessing the normal stem cell properties of self-renewal, the ability to indefinitely generate copies of itself, multipotential differentiation, the capacity to give rise to more differentiated cell types, as well as the additional property of being able to seed and initiate tumorigenesis[51].

Self-renewing multipotent neural stem cells were first appreciated in rat embryonic cortex[52]. It has more recently been appreciated that multi-potent, self-renewing neural stem cells exist in the adult brain[53] and indeed can be isolated from the postnatal cerebellum[54]. It should be noted here that Shh has been shown to be important for driving proliferation and maintenance of self-renewal in the neural stem cells of the postnatal and adult brain[55]. Furthermore, p53 has been observed to be involved in suppression of self-renewal in normal adult neural stem cells[56] as well in medulloblastoma stem cells through interaction with Gli1, the downstream effector of the Shh-signaling pathway[57]. Thus, these cells represent a potential substrate for transformation that could lead to tumors with stem-like characteristics.

This property was first observed within a defined subpopulation of cells in medulloblastomas expressing CD133 (Prominin-1) as a positive surface marker, allowing their fractionation[58]. CD133+ cells can self-renew *in vitro* through forming non-adherent neurospheres and express known neural stem cell markers and genes. Moreover, these cells from primary human medulloblastomas could initiate tumors when transplanted back into immuno-compromised animals. The histopathology of the xenotransplanted tumor recapitulates the heterogeneous cell types of the original human brain tumor, implying multi-potency. Importantly, CD133- cells from the same tumors were unable to re-initiate tumors even when injected in much greater numbers[58]. High level of CD133 expression was also found to be associated with poor prognosis in human medulloblastomas[59].

CD133 surface expression has subsequently been utilized as a means of prospectively isolating cerebellar stem cells from the normal postnatal cerebellum in mice[54]. Further studies utilizing xenograft assays and mouse medulloblastoma models have shown that brain tumor stem cells (BTSCs) may localize to a perivascular niche similar to normal neural stem cells[60] and can be radio-resistant as well[61]. It should be noted that parallel studies initiated within the *Ptc1*-mutant mouse model have proposed CD15 (SSEA1), another known neural stem cell marker[62], as an alternate marker for identifying self-renewing, multipotent, tumor-propagating cells associated with poor prognosis in medulloblastomas[63,64]. These studies have laid the groundwork for carefully investigating and understanding this sub-population of medulloblastoma tumor cells that may represent the driving force behind the formation of more aggressive and treatment refractory tumor-types.

Potential origins of medulloblastoma brain tumor stem cells

In spite of the GCP representing a potential cell-of-origin, the alternate developmental origins of the medulloblastoma tumor-initiating cell remain elusive. As noted previously, the cerebellum primarily originates from two independent germinal zones: the rhombic lip, giving rise to the external granule layer, and the ventricular zone. Mouse models for medulloblastoma have until recently always pointed towards the expansion of a granule cell progenitor as being integral for tumor formation. Mutations within Shh-pathway genes, such as *Ptc1*, *SUFU*, *Smo*, *MYCN*, as well as Shh-independent genes such as *Rb*, *p53*, *Ink4d*, *Lig4* all lead to medulloblastomas with gene expression patterns consistent with the GCP as the cell of origin[41,65]. Moreover, acquisition of a GCP-identity, defined by expression of *Math1*, is an essential step towards tumorigenesis regardless of the timing of Shh-pathway activation in many of these models[66,67]. Lastly, deletion of *Math1* abrogates the ability of the cerebellum to effectively generate granule neurons and prevents medulloblastoma formation[14]. However, given that cells in the EGL represent committed progenitors with limited capacity for self-renewal and multi-potentiality, many questions remain regarding how these cells may acquire more stem-like properties through tumorigenesis.

An alternative to a progenitor cell acquiring stem-like properties is for transformation to take place directly within a tissue stem cell compartment. A number of labs have the taken the approach of enriching for self-renewing cerebellar stem cells through tissue culture and *ex vivo* transforming these cells through either inactivation of the tumor suppressors *Rb* and *p53*[68] or activation of *Myc*[69,70]. Transplantation of the transformed cells has led to aggressive cerebellar tumors that express a gene signature distinct from GCP-derived medulloblastomas and, in the

case of *Myc* activation, is consistent with the Group C subtype. Even so, the exact developmental origin of these cerebellar stem cells is as yet unclear in spite of studies utilizing a number of different neural stem cell markers. CD133+ cells have been observed in the white matter[54] and Sox2+ cells in the Bergmann glia[68,71] of the postnatal cerebellum. Whether the culture-based purification for self-renewing cells is isolating these cells or if transformative events may actually occur within these cell types in the endogenous developmental context remains to be determined.

It is worthwhile mentioning, however, that an alternative medulloblastoma cell of origin has been identified for the Wnt-type tumors through the observation that Beta-catenin (*Ctnnb1*) mutant animals gave rise to cerebellar tumors that originate from the dorsal hindbrain rather than the EGL. These tumors are both anatomically and molecularly distinct from the other subtypes of medulloblastoma[72]. Given that Wnt-type tumors represent the most easily treated medulloblastoma with the best prognosis of the four subtypes, it is my belief that the actual endogenous source of medulloblastoma BTSC driving aggressive disease has yet to elucidated.

Epigenetic regulation of brain tumor stem cells

Epigenetic modifications refer to heritable changes to the genome that do not involve alterations of the underlying nucleotide sequence. Through covalent modifications of DNA as well as of histones in response to both internal and external cues, regions of the genome can be made available for transcription or silenced by epigenetic mechanisms[73,74]. The significance of these pathways in modulating normal development as well as tumor formation has become increasingly appreciated. Within medulloblastoma, tumor-specific methylation changes have been identified in key developmental pathways[75] and genetic analyses in primary tissue samples

have observed that a significant number of tumors contain inactivating mutations within a group of histone modifying enzymes[76]. The lack of overt karyotypic differences between CD133-positive and CD133-negative tumor cell populations[77] suggests that the switch that distinguishes stem from non-stem programs in a transformed cell may exist not on the level of genetics, but through dissimilarities in gene expression and regulation.

The contribution of epigenetic regulation to both normal and malignant stem cell maintenance has been exemplified by work on the polycomb protein Bmi1. A component of the polycomb repressor complex 1, Bmi1 has been shown to be essential for normal cerebellar development through promoting neural stem cell self-renewal[78]. *Bmi1* knockout induces ataxic gait and balance disorders in mice with a severely reduced cerebellum size. *Bmi1* over-expression has been described in a substantial fraction of human Shh-medulloblastomas and shown to be required for the formation of tumors in murine models[79]. Interestingly, Bmi1 has been to shown to directly interact and cooperate with Dnmt1 in gene silencing[80,81], bringing together two distinct mechanisms of epigenetic regulation. Needless to say, the mechanisms by which homeostatic regulation of stem and progenitor populations occurs remain an area of active study.

Research objectives

First, in order to better understand the early influences of tumor suppressor heterozygosity and interactions between tumor suppressor gene mutations on cerebellar development and progression towards malignancy, we conducted studies involving the injection of disease-prone embryonic stem cells carrying $Ptc1^{+/-}$ and $Ptc1^{+/-};p53^{-/-}$ mutations (hereon referred to as Ptc1 and Ptc1;p53 respectively) into wild-type blastocysts to generate somatic mosaic mouse medulloblastoma models. Our observations from these initial experiments laid the groundwork

for appreciating how developmental field cancerization of the cerebellar field by *Ptc1*-mutant cells predisposes an animal for future cerebellar malignancy and identified *Ptc1* loss-of-heterozygosity as a necessary genetic event for tumorigenesis.

Having identified a unique population of aberrantly self-renewing cells in the Ptc1;p53 developing cerebellum and tumor, we then sought to determine if mutations in *Ptc1* and *p53* collaborate in their formation. I observed *Ptc1* haploinsufficiency synergizing with *p53* deficiency to facilitate the complete colonization of the cerebellar stem cell compartment as well as to allow the aberrant persistence of this self-renewing cell population within the pre-malignant cerebellum. These self-renewing cells are similarly unique to the Ptc1;p53 tumors alone and capable of re-initiating robust secondary tumors with a disseminated phenotype. These observations are consistent with identification of a brain tumor stem cell arising through the expansion and malignant transformation of an endogenous tissue stem cell. In seeking to define the developmental origins of the self-renewing cerebellar stem cells through the use of a *Sox2*-reporter animal, I've established Sox2 as a definitive marker for prospective isolation and characterization of the self-renewing tissue stem cell within the normal cerebellum and as a putative marker for brain tumor stem cells within these tumors.

In Chapter 3, I go on to ask whether distinct epigenetic states at the level of DNA methylation exist between the brain tumor stem cell compartment and the bulk tumor, and identify coordinate differences in the promoter methylation of the long non-coding RNA KCNQ1OT1 (Lit1), and Cdkn1c (p57). These changes were functionally corroborated at the level of mRNA expression as well as through RNA-fish experiments demonstrating bi-allelic expression of *Lit1* in the brain

tumor stem cell compartment. Lentiviral-mediated delivery of shRNA constructs was used to characterize the significance of *Lit1* over-expression in brain tumor stem cells wherein knockdown of Lit1 leads to decreased BTSC self-renewal.

The significance of these studies and future directions will be reviewed in the final chapter.

Chapter 2

p53 deficiency facilitates developmental field cancerization preceding malignancy

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Introduction

Cerebellar development is unique within the CNS given that a significant portion of it occurs postnatally. Transformation occurring during this critical developmental window, characterized by extensive cellular proliferation, differentiation, and migration, leads to medulloblastoma, the most common pediatric brain tumor. Within medulloblastoma, it has become appreciated that distinct cells of origin exist[66,70,72] and that directed initiations of tumors from these alternate cellular origins confer characteristic gene expression signatures, histological presentations, and are associated with variable patient prognoses[2,82]. These observations have often relied upon genetic disruption of key tumor suppressor gene pathways such as Rb, p53, and Ptc1, or ectopic activation of known oncogenes such as Myc, Smo, and Ctnnb1 within pre-determined cell lineages[10,33,34,69,70,72,83,84], such that a tumorigenic state is abruptly induced and broadly distributed across the targeted organ. Thus, while these studies have identified key driver mutations and pathways that are necessarily disrupted in the process of tumorigenesis, the importance of earlier events that may occur prior to complete pathway deregulation, such as the effect of the loss of the *first* tumor suppressor allele on cerebellar development and disease susceptibility, remains not well understood. Indeed, reduced allelic expression of the Wnt pathway tumor suppressor *APC* in the absence of other insults is associated with elevated colon cancer risk and non-random allelic loss[85]. In addition, epigenetic modulation precedes Wnt pathway activation in human colorectal cancers[86]. The extent to which graded expression of such morphogenic tumor suppressors, prior to complete loss of function, may facilitate tumor initiation within committed tissue stem or progenitor cells and thus alter disease predisposition is unclear.

Representing nearly 30% of medulloblastomas, Sonic hedgehog (Shh)-responsive tumors are primarily thought to arise through the expansion of granular cell progenitors[66]. Interestingly, incidence of Shh-responsive medulloblastomas exhibits a bimodal distribution wherein tumors occurring during infancy differ significantly from adult medulloblastoma in gene expression pattern and clinical presentation[2]. Similarly, while *Ptc1* mutations are associated with desmoplastic medulloblastomas in humans and mice[2,33], mutations within *p53*, associated with increased tumor grade and relapse in human medulloblastoma patients[37], also significantly increase tumor penetrance and kinetics in $Ptc1^{+/-}$ mice[34]. Consistent with possessing a GCP cell of origin, both $Ptc1^{+/-}$ and $Ptc1^{+/-}$;$p53^{-/-}$ (hereafter referred to as Ptc1 and Ptc1;p53, respectively) medulloblastomas have been previously described as having similar expression patterns as each other and exhibiting Shh-dependency[42,43,65]. Even so, while small-molecule inhibition of Shh-pathway activity dramatically reduced metastatic tumor burden in a patient, the occurrence of relapse in both human[44] and mouse models[45] points toward the existence of an unappreciated cellular reserve that is independent of Shh and capable of re-initiating tumorigenesis.

Disseminated tumor growth, a marker of particularly poor prognosis and aggressive medulloblastoma, has recently been shown to be inducible from both *Ptc1* and *p53* mutant backgrounds[87]. Moreover, metastatic human medulloblastomas share gene expression profiles with the early postnatal murine cerebellum[88] during a time when multipotent cerebellar stem cells as well as GCPs are present[54]. Consistent with a cancer stem cell model for medulloblastomas, elevated levels of CD133[59] and CD15[64] (also known as Lewis X antigen and SSEA1) stem cell markers have been associated with poor prognosis, and murine models for the most aggressive medulloblastoma subtype have recently been generated through forced

overexpression of Myc within cerebellar stem cells isolated from p53-deficient animals[69,70]. However, the endogenous context by which malignant transformation of a tissue stem cell occurs remains to be elucidated.

Here we show that a genetic interaction between the *Ptc1* and *p53* transforms cerebellar stem cells during development and leads to an aggressive tumor with stem-like characteristics that are not seen in Ptc1-deficient medulloblastomas with functional p53. Using somatic mosaic models of medulloblastoma, we ascribe synergism between haploinsufficiency of Ptc1 combined with p53 loss that causes an unexpected and fully penetrant developmental seeding of the cerebellar field as well as the cerebellar stem cell field. This pre-malignant cancerization of the stem cell field results in aberrant, persistent tissue stem cells and tumors bearing a unique population of tumor-initiating cells expressing both cerebellar stem and granule cell progenitor markers. Through orthotopic transplantation, we found this population capable of generating robust cerebellar tumors with metastatic properties. These data support a model wherein developmental cancerization of a tissue stem cell field observed within the Ptc1;p53 genotype profoundly influences the character of the tumor that is eventually formed.

Results

Aggressive, diffuse medulloblastomas arise from a mutant developmental field

While conditional genetic models give rise to highly penetrant and rapidly inducible tumors, these approaches may overlook important key developmental events that occur prior to overt tumorigenesis. Furthermore, given that the majority of human medulloblastomas arise from *de novo* somatic mutations, early interactions between wild-type and mutant cells may be particularly important in facilitating tumorigenesis. Thus, in studying the role of Ptc1 and p53 tumor suppressors in cerebellar development and medulloblastoma formation, we utilized a somatic mosaic approach that establishes an *in vivo* competition between the wild-type and mutant ES genotypes, enables cell marking for lineage tracing experiments within the brain[89], and is amenable to generating developmentally synchronized mutant and control cohorts. To achieve this, we injected disease-prone embryonic stem (ES) cells with $Ptc1^{lacZ/+}$, $p53^{-/-}$ or $Ptc1^{lacZ/+}$;$p53^{-/-}$ (abbreviated as Ptc1, p53, and Ptc1;p53, respectively) genotypes into wild-type blastocyst to generate chimeric animals (illustrated in Figure 2-1) as a means of assessing the developmental and tumorigenic potential of cells carrying these mutations.

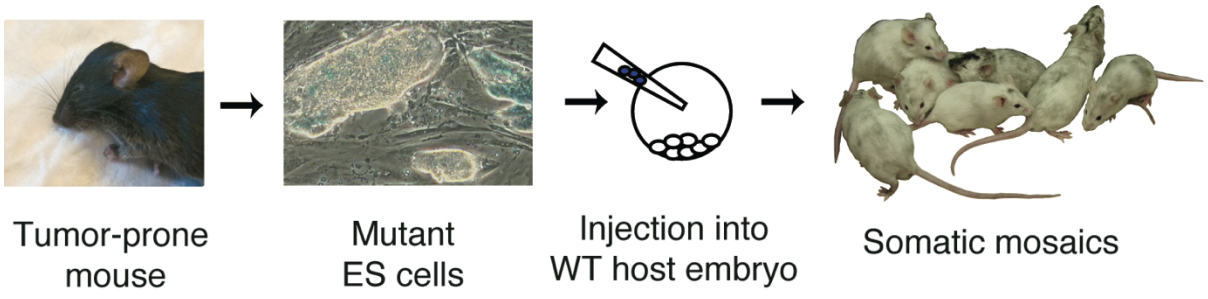

Figure 2-1 | Establishing a somatic mosaic model for medulloblastoma
Schematic illustrates the process by which disease-prone ES cells are derived from animals and utilized for production of somatic mosaic animals.

We observed that chimeric Ptc1 and Ptc1;p53 animals were able to recapitulate tumor histology, kinetics, and incidence seen in bred animals. Interestingly, while no differences in gene expression have been previously described between these two medulloblastoma models, we immediately noticed differences in the histological presentation of the tumors: Ptc1 chimeras usually showed focal lesions that displace adjacent folia whereas Ptc1;p53 chimeras tended to develop diffuse medulloblastomas that invade between the folia and extend over the majority of the outer cerebellum (Figure 2-2).

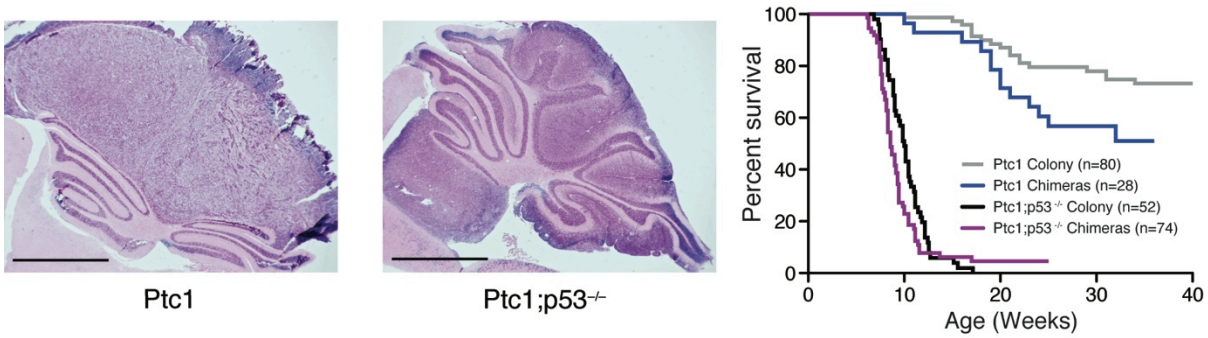

Figure 2-2 | Ptc1 and Ptc1;p53 chimeras recapitulate tumor phenotype
Representative hematoxylin and eosin stained sections from chimeric Ptc1 and Ptc1;p53 cerebellar tumors demonstrate focal Ptc1 tumors and diffuse, infiltrating Ptc1;p53 tumors. Scale bars, 2mm. The Kaplan-Meier survival curves show chimeras recapitulate the kinetics and incidence of medulloblastoma formation seen in genetically bred animals.

Expression of CD15 predicts poor prognosis in human medulloblastoma and is a marker for self-renewal in normal and tumor-derived neural stem cells[64]. Moreover, it has recently been appreciated that expression of chemokine receptor CD184 (CXCR4), already known to be involved in facilitating GCP migration and proliferation[24,26], defines a distinct molecular subgroup of Shh-driven medulloblastoma[25]. In looking to begin characterization of molecular differences, we first observed a statistically significant enrichment for cells expressing CD15 and CD184 in Ptc1;p53 tumors (Figure 2-3). These data are consistent with the more rapid onset and

increased penetrance as well as diffuse tumor presentation of Ptc1;p53 tumors representing a distinct and more malignant type of tumor from those found in the Ptc1 animals.

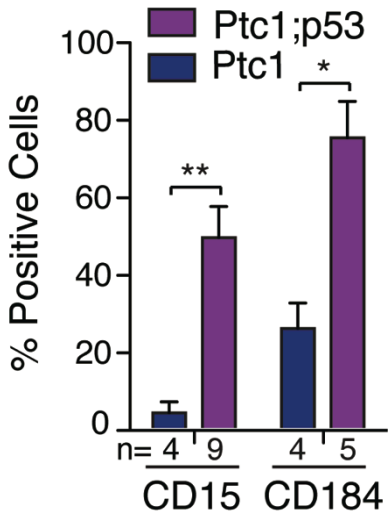

Figure 2-3 | Ptc1;p53 tumors have elevated levels of neural stem cell markers CD15 and CD184
Flow cytometric analyses of primary brain tumors for Ptc1 and Ptc1;p53 animals reveal a significant difference in the surface expression of the neural stem cell markers CD15 and CD184.

While forced activation of Shh signaling is sufficient to initiate cerebellar tumorigenesis[84,90], conflicting observations on the genetic and epigenetic status of the wild-type *Ptc1* allele in Ptc1 and Ptc1;p53 tumors have obscured our understanding of the required changes involved in tumorigenesis[42,63,91]. Resolving this discrepancy is necessary for identifying and tracking tumorigenic cells. We observed *Ptc1* loss-of-heterozygousity (LOH) via direct measurement of the wild-type *Ptc1* allele by qPCR in 17 of 18 Ptc1 and all 16 Ptc1;p53 tumors but not in any of the 11 older asymptomatic Ptc1 cerebella examined (Figure 2-4). These results support *Ptc1* LOH as a common genetic alteration underlying malignant transformation in these tumors, providing a marker for tumor-initiating events.

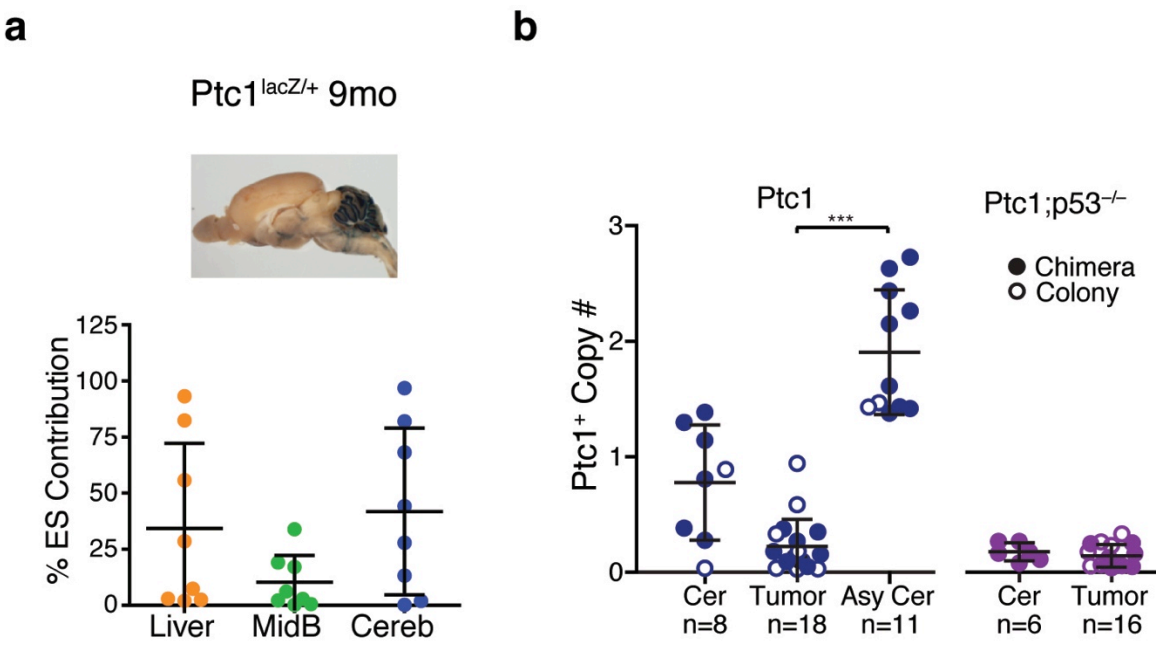

Figure 2-4 | Ptc1 loss-of-heterozygosity occurs in Ptc1 and Ptc1;p53 medulloblastomas
a, Nine-month old, asymptomatic Ptc1 animals demonstrate that contribution bias of Ptc1 cells is independent of tumorigenesis. b, Quantification of the wild-type *Ptc1* allele (*Ptc1*$^{+}$) in end-point Ptc1 and Ptc1;p53 mice using qPCR demonstrates loss of *Ptc1*$^{+}$ specifically in tumors but not paired midbrain samples or in cerebella of old (9 months), asymptomatic Ptc1 mice (Asy Cer).

Surprisingly, we found that although the mutant ES genotype distinguished the tumor classes, there was no correlation between the extent of somatic contribution as assessed by coat color and the average endpoint age for either Ptc1 or Ptc1;p53 genotypes (Figure 2-5). Within the brain, variation in chimerism was detected outside the cerebellum, but tumors and cerebellar tissue appeared to predominantly consist of Ptc1;p53 mutant cells as assessed by *Ptc1*$^{lacZ/+}$ expression, *p53* genotyping, and genomic qPCR to quantify *neo*:*gapdh* copy number (Figure 2-6a). Tumors and cerebellar tissues were entirely comprised of Ptc1;p53 mutant cells in nearly all animals examined (Figure 2-6c), whereas variable contributions of Ptc1;p53 mutant cells were seen in liver and "midbrain" (defined in Methods). These results raised the question whether the tumor suppressor mutations enable cells to favorably respond to developmental patterning cues leading

to cerebellar contribution at the exclusion of wild-type host tissue, or if the bias in contribution is a consequence of tumorigenic selection.

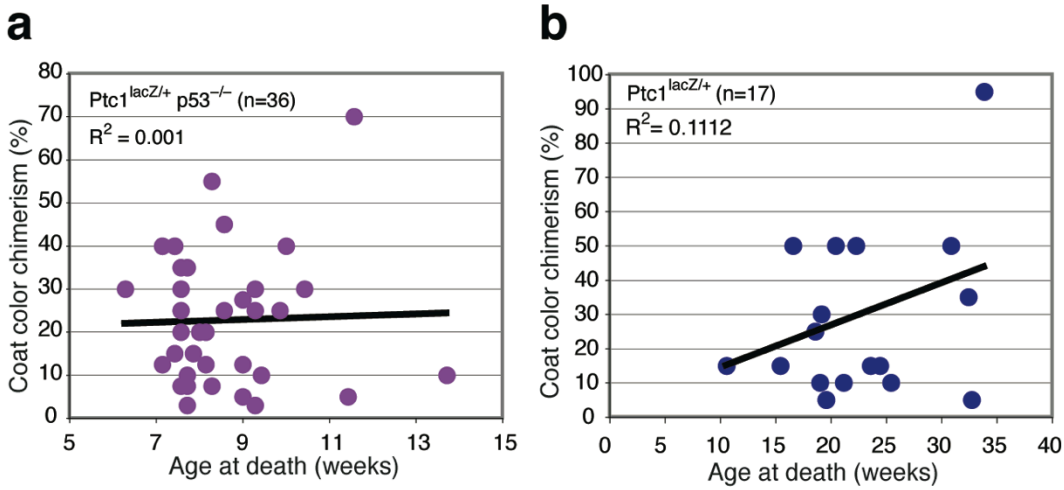

Figure 2-5 | Variation in body chimerism does not predict age of morbidity
Degree of overall somatic chimerism as determined by coat color contribution is not reflective of age of death in either Ptc1;p53 (a) or Ptc1 (b) chimeras.

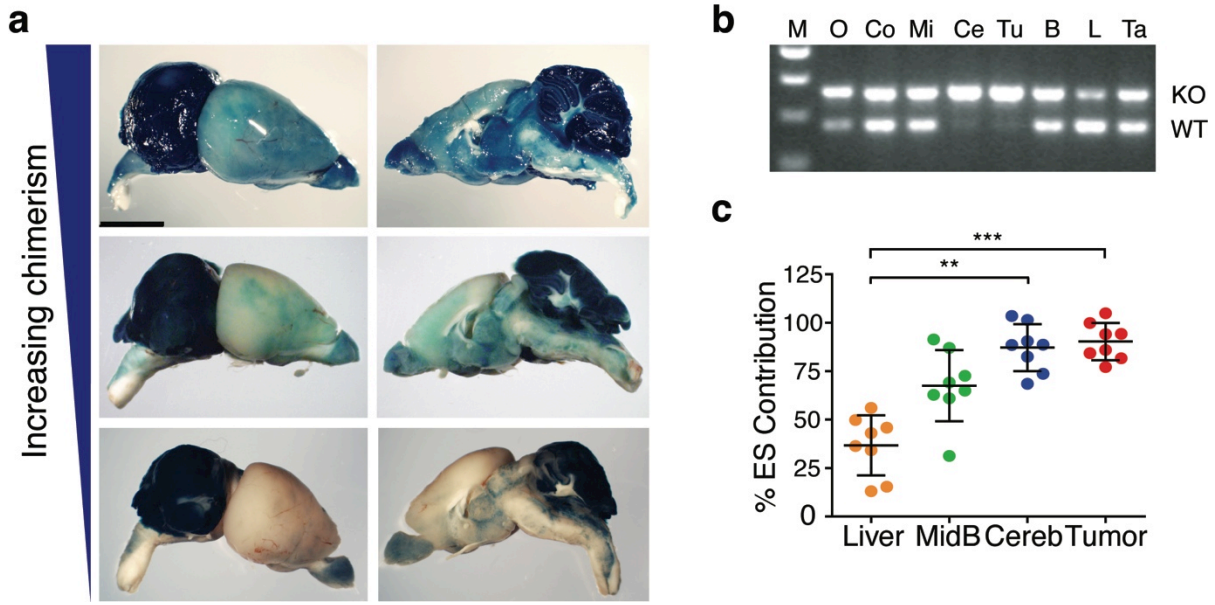

Figure 2-6 | Ptc1;p53 chimeras exhibit a contribution bias to cerebellar tumors
a, Representative X-Gal-stained whole mounts from endpoint Ptc1;p53 chimeric animals demonstrate preferential competitive colonization of both cerebellar and olfactory compartments (n= 16). b, Genotype analysis for mutant ES-derived and wild-type host derived p53 alleles shows the cerebellum and tumor are extensively mutant-derived (n= 5) c, Quantified genomic contribution using neo/gapdh ratios determined by qPCR (n=8).

In order to examine developmental bias independent of tumorigenesis, we initially measured cerebellar contribution in three-week old Ptc1 and Ptc1;p53 chimeric animals, a time when normal cerebellar development is considered largely complete but well before the occurrence of tumors in either genotype. Interestingly, the cerebella of these young animals also showed strongly biased contribution towards the mutant genotype as observed by *lacZ* expression (Figure 2-7a, n= 5) and *p53* PCR (Figure 2-7b, n=3), despite a range in overall brain and somatic chimerism. To parse apart the roles of *Ptc1* and *p53* mutations in driving colonization of the cerebellar field, we additionally examined three-week *p53*-null chimeras. Measurement of *neo*:*gapdh* by qPCR similarly showed 100% skewed contribution of Ptc1;p53 mutant cells and a significant enrichment for contribution in the Ptc1 genotype relative to cells deficient for p53 alone (Figure 2-7c). These data are consistent with cerebellar enrichment occurring secondary to an increased ability of the mutant cells to respond to developmental patterning cues rather than tumorigenic selection and suggest that Ptc1-haploinsufficiency is driving this selection.

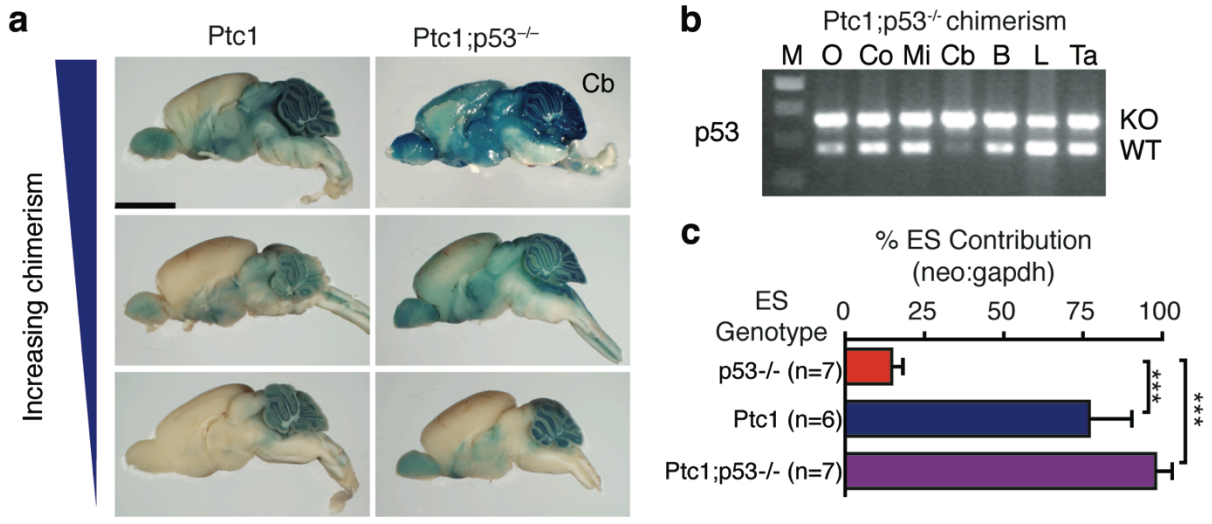

Figure 2-7 | Ptc1 haploinsuffiency drives colonization of pre-malignant cerebellum
Cerebella of three-week old Ptc1 and Ptc1;p53 chimeric animals are largely mutant-derived as seen by X-gal staining (**a**) and gel-based PCR for the *p53* alleles (**b**). **c**, qPCR copy number analysis of *neo*:*gapdh* in three-week chimeric cerebellum demonstrates significant enrichment of Ptc1 cells ($p<0.001$) and complete colonization by Ptc1;p53 cells ($p<0.001$) compared to p53 knockout chimeras.

To further investigate this hypothesis, we considered that colonization of the cerebellar anlage by Ptc1;p53 mutant cells may be due to an enhanced responsiveness to the morphogen Shh. To ascertain this, we looked first at surface expression of CD184, a known Shh-responsive marker, and observed an eight-fold increase in CD184+ cells in the Ptc1;p53 genotype relative to Ptc1, p53, and wild-type in three-week animals (Figure 2-8a). We also observed significantly increased expression of Shh pathway genes *Math1, Gli1,* and *Gli*2 at the mRNA level (Figure 2-8b). These studies were further substantiated via an *in vivo* competition assay involving co-injection of both Ptc1 and Ptc1;p53 mutant ES cells into wild-type blastocysts. In order to distinguish between the different mutant genotypes *in vivo*, we additionally marked Ptc1 ES cells with an eGFP reporter and Ptc1;p53 ES cells with an mCherry fluorescent reporter. Here we observed that Ptc1;p53 cells out-competed even Ptc1 cells in the colonization of the cerebellum as well as the dentate gyrus (Figure 2-8c).

Given that we observed complete colonization of the cerebellar fields and elevated Shh signaling within the three-week Ptc1;p53 cerebellum, we now asked if *Ptc1* LOH had already occurred at this early time-point. Weanling Ptc1 chimeras showed no evidence of LOH and Ptc1;p53 chimeras showed only modest reduction in average copy number (Figure 2-9). Thus, loss of *p53* synergizes with *Ptc1* haploinsufficiency to convey hyper-responsiveness to Shh-signaling and facilitate the establishment of not merely a pre-malignant field but an entire organ system poised for future malignancy. We found these observations to be reminiscent of field cancerization, a process that gives rise to polyclonal epithelial tumors often associated with p53 mutations[92].

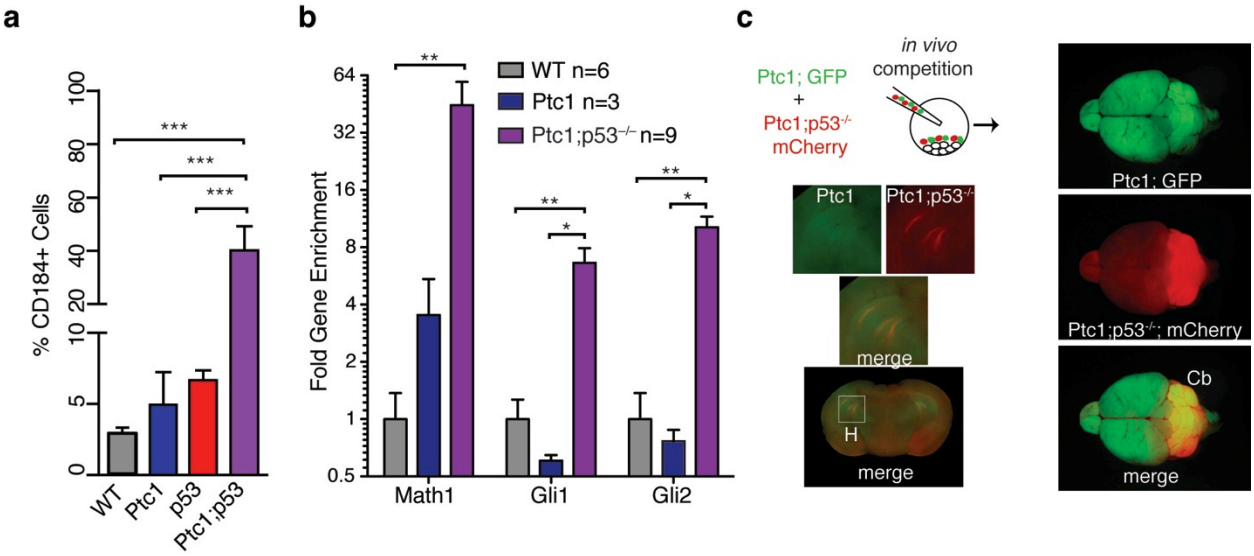

Figure 2-8 | Increased Shh activity in Ptc1;p53 3-week cerebella facilitates tissue colonization
a, Expression of the cell surface marker CD184 is significantly increased in Ptc1;p53 three-week cerebellar tissue (n=4), over that of wild-type (n=7), Ptc1 (n=5), and p53 (n=3) by flow cytometric analyses. b, PCR analyses of mRNA from three-week cerebella of the indicated genotypes show significantly increased *Math1* expression as well as elevated levels of the Shh-effectors *Gli1* and *Gli2* for the Ptc1;p53 genotype. c, Fluorescent images from double chimeras derived from both Ptc1 and Ptc1;p53 mutant cells shows increased ability of the double-mutant cells to occupy the dentate gyrus as well as the cerebellum.

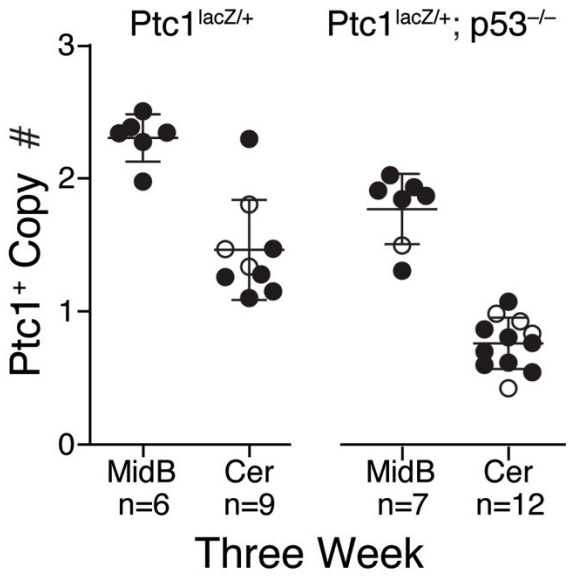

Figure 2-9 | Ptc1 wild-type allele is maintained in weanling animals prior to tumorigenesis
Quantification of the wild-type *Ptc1* allele by qPCR in three-week old cerebella from Ptc1 and Ptc1;p53 animals supports retention of the allele with a slight reduction detected in Ptch;p53 prior to tumorigenesis.

Interaction between Ptc1 and p53 facilitate cerebellar stem cell field colonization

While Ptc1 and Ptc1;p53 tumors are widely regarded as interchangeable disease models[63,65], our observations thus far are consistent with the Ptc1;p53 medulloblastoma representing a distinct and more malignant tumor-type. Recent work by others in the field have shown that cerebellar stem cells are potential cells of origins for aggressive medulloblastoma[68-70]. Moreover, independent roles have been ascribed to Shh signaling[55] and p53[56,93,94] in cell fate and self-renewal decisions within neural stem cells. During normal cerebellar development, cerebellar stem cells with multi-lineage potency and functional properties of neural stem cells are abundant at postnatal day 7 (p7), after which time stem cell numbers diminish in the post-mitotic organ[54]. As such, we now sought to address the influence of Ptc1, p53, and Ptc1;p53 genotypes on early cerebellar development. To achieve this, we examined markers of tissue contribution and stem cell field colonization in chimeras (Figure 2-10a).

As in tumor-bearing and weanling animals, developmental bias of Ptc1;p53 cells was observed by p7 with uniformly high contribution to the cerebellum seen across a range of brain chimerism where wild-type control chimeras showed distributed contribution throughout the brain without particular emphasis in the cerebellum (Figure 2-10b). To measure stem cell field cancerization, clonogenic neurosphere cultures were used to select for cells with neural stem-cell properties. Individual neurospheres from these low-density cultures were genotyped for host versus ES cell origin, which revealed 100% colonization of the stem cell population by Ptc1;p53 cells whereas Ptc1 cells contributed 67% and wild-type chimeras showed only 18% contribution (Figure 2-10d). Thus, while the Ptc1 genotype drives extensive colonization of cerebellar tissue in

mosaic animals, the Ptc1;p53 genotype facilitates complete developmental field cancerization of the stem cell niche as well.

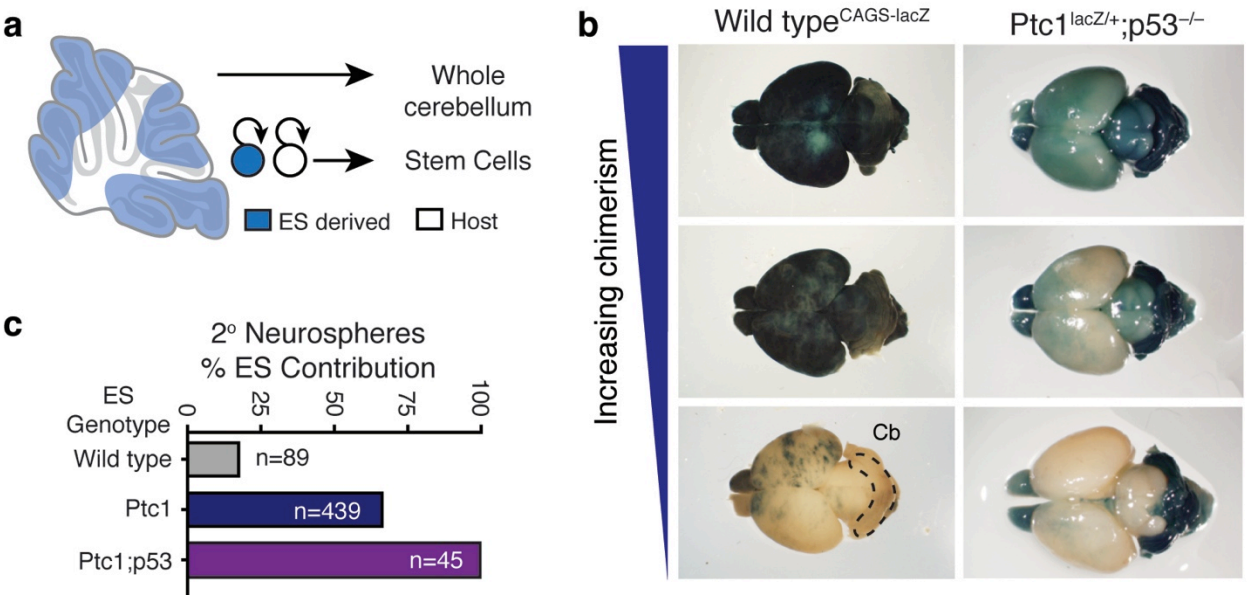

Figure 2-10 | Ptc1 synergizes with p53 deficiency to drive cerebellar stem cell field cancerization **a,** Schematic of analysis of mutant ES cell contribution to whole cerebellum and cell-type subsets. **b,** *Ptc1*^{lacZ} expression in p7 chimeric brains shown by X-gal staining demonstrates a contribution bias of Ptc1;p53 cells to the cerebellum (dashed line) not seen in wild type ES cells expressing *CAGGS-lacZ*. **c**, Genotyping of clonal cerebellar stem cells (from low-density neurosphere cultures) derived from chimeric p7 cerebella shows elevated contribution by Ptc1 and complete colonization by Ptc1;p53 cells as compared to wild type ES chimeras.

Ptc1 and *p53* loss synergize in cerebellar and tumor stem-cell self-renewal

Persistent developmental rests that occasionally progress to tumorigenesis frequently occur in Ptc1 animals[29,95]. The stem cell field colonization seen in Ptc1 and Ptc1;p53 p7 chimeras prompted us to ask whether stem-like cells persist beyond the normal 3-week developmental window in Ptc1 and Ptc1;p53 cerebella or can be found in tumors through culturing cerebellar cells under clonogenic conditions. Significant but low numbers of self-renewing cells were detected in 12 out of 17 *p53*-deficient animals, whereas stem cells were absent in 100% of wild-type controls (n=9). In contrast, the majority of Ptc1 showed undetectable self-renewal and p53 cerebella only modest levels (Figure 2-11a). These observations were recapitulated in adult animals where Ptc1;p53 tumors likewise showed a 100-fold increase in self-renewing cells relative to Ptc1 tumors (Figure 2-11b). Age-matched wild-type controls and asymptomatic Ptc1 animals showed no measurable self-renewing cells in this assay (data not shown).

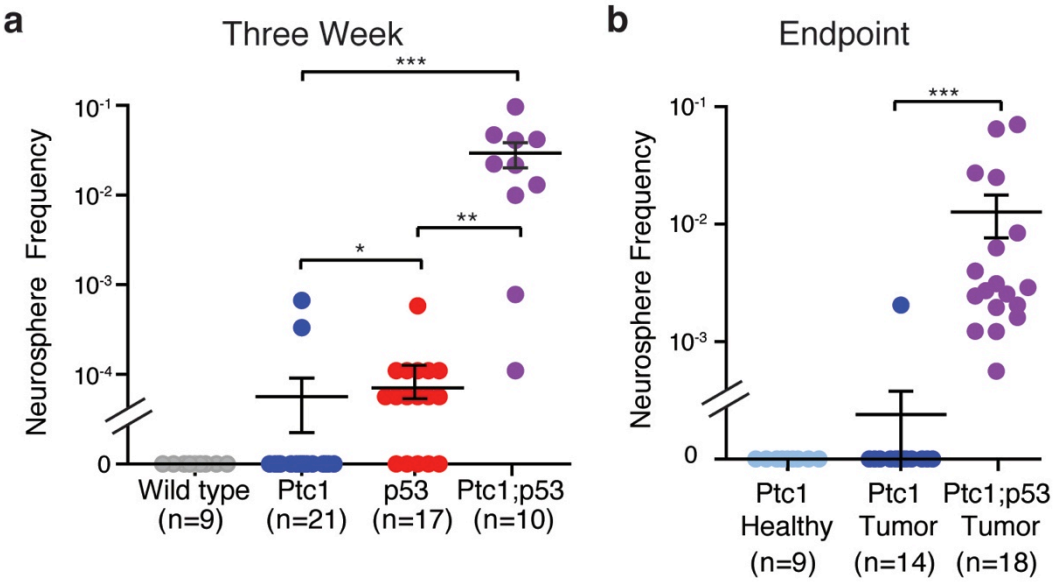

Figure 2-11 | Ptc1;p53 animals possess aberrant tissue and tumor-derived stem cells
a, Clonogenic Ptc1;p53 self-renewing cells are developmentally expanded and aberrantly persistent prior to tumor formation, and p53 cerebella show an elevated basal level of neurosphere forming ability relative to Ptc1 chimeras. **b,** Clonogenic self-renewing cells are significantly elevated in Ptc1;p53 tumors over Ptc1 tumors.

In order to functionally compare 3-week aberrant tissue stem cell with the tumor-derived stem cells, we looked first at CD15 and CD184 marker expression and observed a significant increase in cells positive for both markers in tumor versus 3-week cerebellar samples (Figure 2-12a). Quantitative genotyping of clonal neurospheres revealed *Ptc1* LOH in all tumor-derived Ptc1;p53 neurospheres, but no LOH was observed in 3-week Ptc1;p53 clones (Figure 2-12b), supporting the idea that the up-regulation of these markers follows *Ptc1* LOH and marks malignant progression. Accordingly, we found that Ptc1;p53 3-week cerebellar stem cells, unlike their tumor-derived counterparts, were unable to resist differentiation and retain high levels of *Sox2* expression following growth factor withdrawal (Figure 2-12c). Therefore, the aberrant self-renewing cerebellar stem cells in 3-week Ptc1;p53 animals retain differentiation capacity that appears diminished upon *Ptc1* LOH and tumorigenesis.

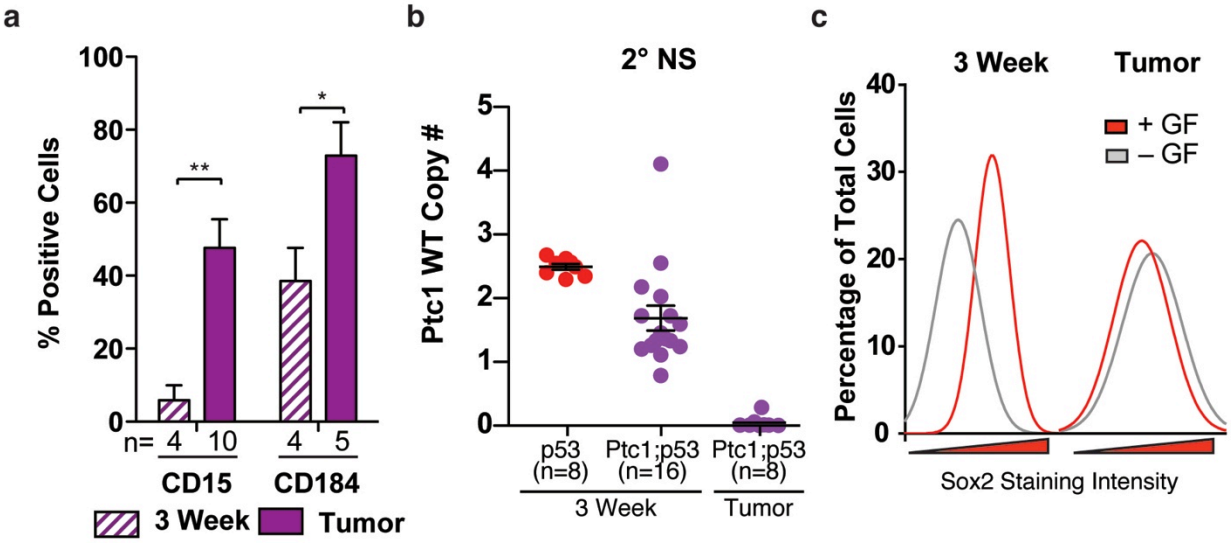

Figure 2-12 | Aberrant tissue stem cells differ from brain tumor stem cells
a, Surface expressions of both CD15 and CD184 show significantly increased expression in Ptc1;p53 tumors as compared to 3-week pre-malignant cerebella as assessed by flow cytometry. b, Copy number quantification of *Ptc1*$^{+}$ shows retention in clonal neurospheres from 3-week animals and LOH in tumor-derived cultures. c, 3-week clonal neurospheres retain a differentiation response and down-regulate Sox2 following growth factor withdrawal, while tumor-derived cells maintain high levels of Sox2 in the absence of growth factor stimulation.

Ptc1;p53 tumor stem cells initiate metastatic medulloblastoma

Mouse models of medulloblastoma have shown that different populations of neural progenitors or stem cells can be transformed through activation of distinct signaling pathways[69,70,72,90]. Given their gene expression profile and dependency on Shh-pathway activity, Shh-driven tumors are widely thought to arise through transformation of granule cell progenitors[66]. In contrast to these studies, we now sought to determine whether aberrant cerebellar stem cells or tumor stem cells from Ptc1;p53 animals have tumor initiating potential.

To examine these functional properties, brain tumor stem cell (BTSC) lines were established through weekly, serial passaging of tumor neurospheres in the presence of bFGF and EGF. During this period of time, an 80-fold increase in clonogenic self-renewal was observed in the BTSC lines compared to primary cultures, as measured by quantitative limiting dilution assays (Figure 2-13a). Consistent with the enrichment for BTSC self-renewal activity, progressive increases in Sox2 levels were observed via immunofluorescence (Figure 2-13b, c). Clonal BTSC lines were generated from single neurospheres to demonstrate long-term self-renewing activity of these cells. Surface marker analyses of clonal derivatives from two independent tumor lines revealed considerable heterogeneity of marker expression for the neural stem cell markers CD15, CD133, and CD184 (Figure 2-13e). In contrast, high levels of Sox2 mRNA were observed in all BTSC lines. The clonal line B1 with uniformly high levels of all three markers was selected for comparison to primary tumors for the subsequent transplantation studies.

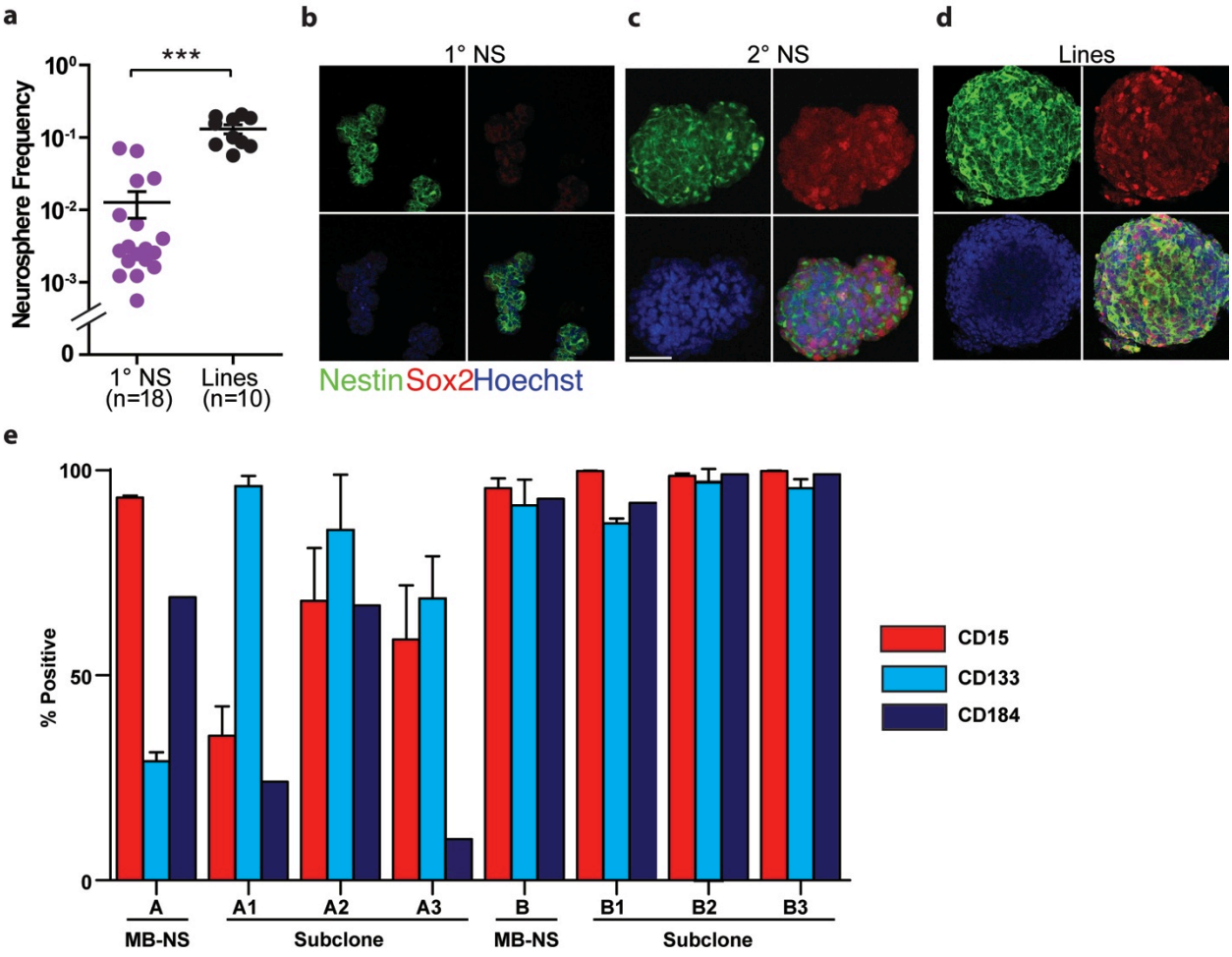

Figure 2-13 | In vitro cultures enriches for Sox2-positive self-renewing cells
a, Neurosphere forming potential is significantly increased ($p<0.0001$) in the established neurosphere lines over that found in primary neurospheres from tumors. Immunofluorescent staining for the neural stem cell markers Sox2 (red) and Nestin (green) in neurospheres derived from primary and secondary cultures of ptc1;p53 tumors (b, c) and clonally-derived lines (d) shows persistent Nestin expression and selection for increased Sox2 expression in culture. e, Surface marker analyses of long-term cultured medulloblastoma neurosphere lines (MB-NS) and FACS isolated single-cell subclones. Heterogeneity in CD15, CD133 and CD184 levels is seen among the line and subclones from tumor A whereas uniform high expression of all three markers was seen in the Tumor B line and subclones.

Secondary tumor initiation was determined by intracerebellar injection of 5 x 10^4 cells via stereotactic surgery. Animals were observed closely until development of signs of morbidity before being euthanized. Consistent with elevated stem cell marker expression and self-renewal of culture-selected BTSC lines, median survival was significantly shorter than that found from comparable injections of primary tumor cells (Figure 2-14a). Animals injected with BTSC lines

developed malignant growths in the cerebellum and often in the olfactory bulb, as shown by LacZ staining (Figure 2-14b). This unexpected observation was recapitulated through injection of primary, unfractionated tumor cells (Figure 2-14c), indicating that the disseminated tumor phenotype is intrinsic to the Ptc1;p53 tumors and not acquired through prolonged time in culture. By contrast, primary tissue from 3-week cerebellum engrafted in only 21% of recipients within three months, whereas 3-week culture-selected cerebellar stem cells failed to exhibit any tumor engraftment up to 15 weeks. Given that the 3-week cerebellum demonstrated levels of self-renewing activity comparable to the BTSC (Figure 2-11) but *Ptc1* LOH was not detectable in either the cerebellar tissue (Figure 2-9) or the self-renewing stem cells (Figure 2-12b), these results support *Ptc1* LOH as a necessary event for tumorigenesis.

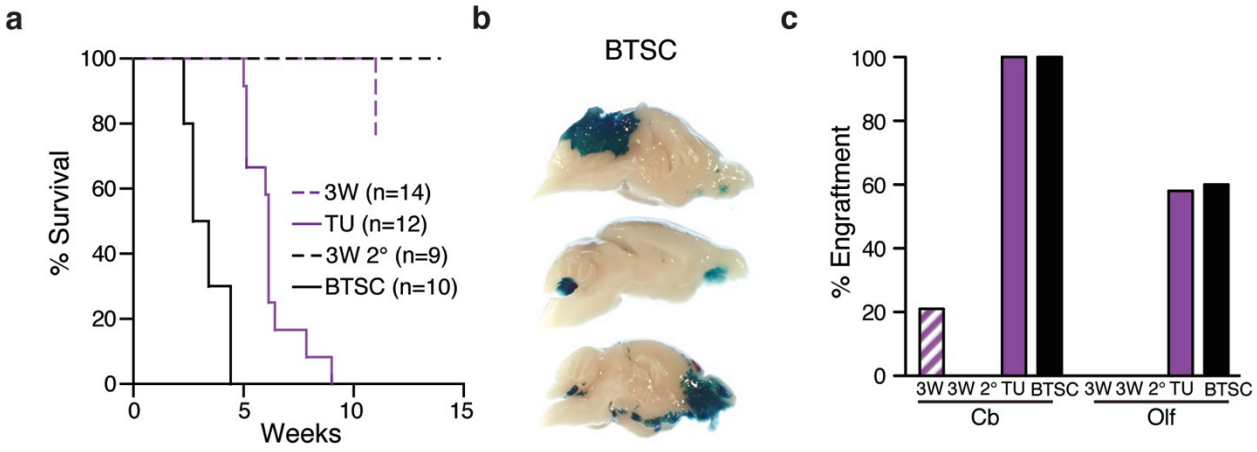

Figure 2-14 | Brain tumor stem cell gives rise to rapid, disseminated medulloblastoma
a, Kaplan-Meier tumor-free survival curves of mice injected with $5x10^4$ cells from unfractionated 3-week cerebella (3W), tumors (TU), secondary clonogenic stem cells from 3-week cerebella (3W 2°), and brain tumor stem cell lines (BTSC). b, Sagittal sections of X-gal stained brains from BTSC xenograft injections. c, Both primary, unfractionated tumors as well as BTSC lines are able to give rise to cerebellar and disseminated disease.

High CD184 expression is associated with disseminated tumor phenotype

While the observation of disseminated disease is generally associated with poor prognosis[2], there exist few mouse models for studying disseminated medulloblastomas[41]. Interestingly, we noted that the disseminated phenotype observed in primary tumor injections could be tracked back to cells isolated from the same original tumor (Figure 2-15a, 'Tumor 2'). We had earlier reported high levels of CD184 expression in the developing and tumor-bearing cerebellum as well as clonal brain tumor stem cells of Ptc1;p53 animals. Retrospective CD184 marker analyses of cultured cells from the injected tumors by flow cytometry revealed low marker expression in Tumor 1 and high marker expression in Tumor 2. Fractionation of bulk tumor cells by CD184 surface expression did not enrich for self-renewing potential. Given the known role of this chemokine receptor in directing migration and driving proliferation of cerebellar progenitors[96], these data are suggestive that high CD184 levels convey metastatic properties onto Ptc1;p53-derived BTSC.

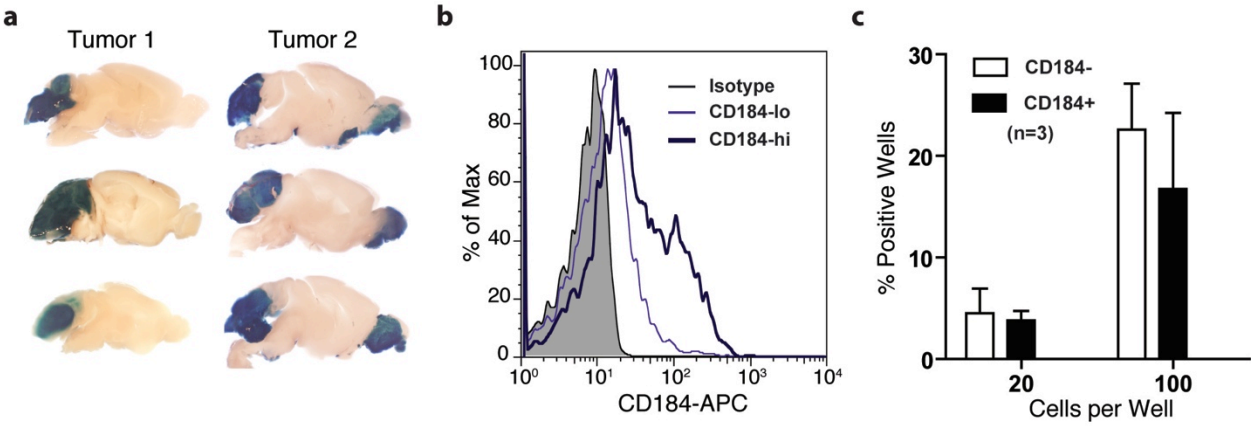

Figure 2-15 | CD184 expression levels reflect metastatic potential
a, Injection of unfractionated primary tumor cells from independent tumors reproducibly engraft in the cerebellum alone (Tumor 1) or both cerebellar and olfactory regions (Tumor 2). b, Retrospective FACS analyses of CD184 expression from passage 4 bulk neurosphere cultures derived from Ptc1;p53 tumors used for primary tissue injections in part a. CD184-hi profiles correspond to tumors that yielded locally metastatic secondary tumors. c, Limiting dilution clonogenicity assays show that self-renewing potential is not fractionated by surface expression of CD184.

Sox2 expression functionally defines self-renewing cerebellar stem cells from progenitor cells

Mapping of gene expression data from metastatic human medulloblastoma samples onto mouse cerebellar development shows the greatest overlap with postnatal day 5[88], a time-point when multipotent, self-renewing cerebellar stem cells are known to exist and are readily abundant. While concordant gene expression profiles between Ptc1 and Ptc1;p53 medulloblastomas have suggested that GCP markers define these tumors, the observation of a definable, self-renewing population of stem-like cells led us to ask if the tumor-initiating cells in the Ptc1;p53 medulloblastoma more closely resemble cerebellar stem cells or granular cell progenitors.

The most frequently utilized marker for prospectively identifying and isolating self-renewing cerebellar stem cells has been the surface marker CD133, also known as Prominin-1. However, this approach requires simultaneous selection against numerous lineage-specific markers in order to achieve a significant enrichment for self-renewing stem cells. It has also been shown that a significant fraction of multipotent, clonogenic neural stem cells lacks expression of both CD133 and CD15, another commonly used marker[97]. Even within the highly self-renewing clonogenic Ptc1;p53 BTSC lines, we observed significant heterogeneity for these surface markers. However, the transcription factor Sox2 has been shown to play a vital role in marking and maintaining adult stem and progenitor cells in a variety of tissues[98] including the CNS[99,100], and its expression is associated with worse prognosis in human medulloblastomas[68]. Within the mature cerebellum, Sox2-staining has been observed in the white-matter as well as in Bergmann glial cells within the Purkinje cell layer but little *in vivo* characterization has been performed[68,101]. Given the

uniformly high levels of *Sox2* expression we observed in the Ptc1;p53 BTSC, we thought to test whether Sox2 would function as a definitive cerebellar stem cell marker.

To approach this question, we utilized a previously characterized *Sox2-GFP* knock-in reporter animal[98,99] and immediately observed a distinct population of GFP-positive cells constituting 6.5% of cells within the p7 cerebella ([59,98,99,102]a). While concurrent surface marker analyses for CD15 and CD133 demonstrated a similar frequency of positive cells (6.4% and 4.9%, data not shown), cells expressing these neural stem cell markers were highly enriched in the Sox2+ fraction of the cerebellum (Figure 2-16b). In clonogenic neurosphere cultures of unfractionated cerebellum, we observed that all neurospheres were uniformly positive for GFP (Figure 2-16c). Moreover, fractionation based on GFP-marker expression permitted the absolute isolation of self-renewing activity within the Sox2+ fraction (Figure 2-16d); plating even $2x10^5$ Sox2-negative cells yielded no neurospheres in this assay (data not shown). Finally, consistent with GFP-fractionation separating cerebellar stem cells from granule cell progenitors and the fact that these two populations are thought to express distinct gene signatures, we observed 10-fold enrichment for *Sox2* accompanied by 10-fold depletion in *Math1* expression within GFP-positive cells as measured by qPCR (Figure 2-16e). Taken together, our data support Sox2 as a definitive marker for the self-renewing cerebellar stem cell within the normal, developing cerebellum and offers a simple yet effective approach for the further isolation and study of these cells.

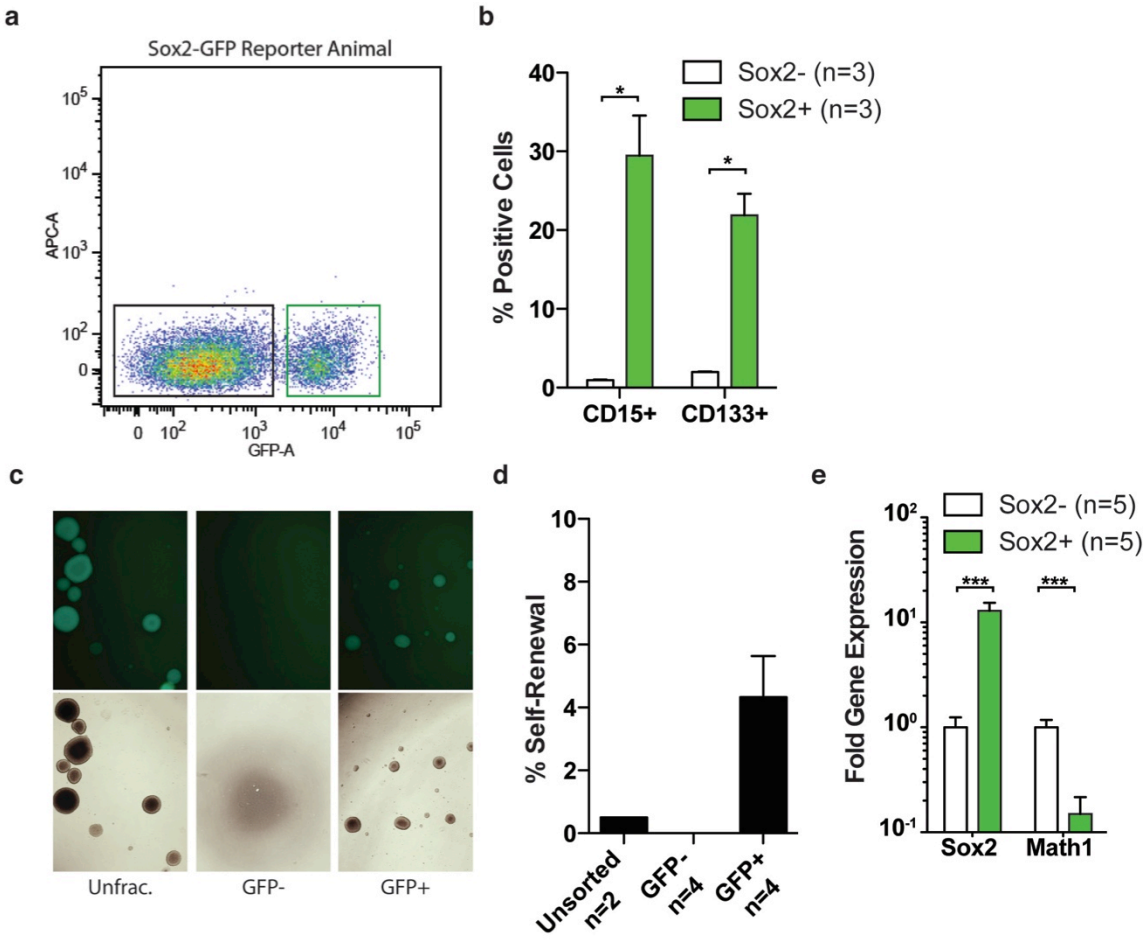

Figure 2-16 | Sox2 marks self-renewing cerebellar stem cells in the postnatal cerebellum
a, Analysis of dissociated cerebellar cells by flow cytometry shows an easily identifiable population of GFP-positive cells. b, Sox2-positive cells are enriched for expression of other neural stem cell markers. c, Fluorescent as well as bright-field images from low-density neurosphere culture of unfractionated and as well as GFP- and GFP+ cells demonstrate marked enrichment for self-renewing cells in the GFP+ fraction, quantified in (d). e, GFP-positive cells highly express *Sox2*, not *Math1*.

Ptc1;p53 BTSC maintain Shh-signaling in spite of differentiation cues

Having shown that within the normal cerebellum Sox2-positive cerebellar stem cells are distinct from *Math1*-expressing granule cell progenitors, we now sought to extend these observations to the medulloblastoma disease models by quantitative comparison of gene expressions for a small subset of GCP-signature genes (*Math1*, *Gli1*, and *Gli2*) and CbSC-signature genes (*Sox2*, *Hes1*). We found that GFP-fractionation of wild-type p7 cerebellum demonstrated enrichment for the

GCP-signature in the Sox2-negative fraction and CSC-signature in the Sox2-positive fraction. Consistent with a bFGF-dependent mechanism for blocking Shh-signaling and inducing GCP differentiation[103], we observed that culturing wild-type cerebellar stem cells in the presence of bFGF and EGF results in abrogation of the GCP-signature but maintenance of the CbSC signature (Figure 2-17, left panel). Interestingly, even though Ptc1;p53 cerebella already show aberrant expression of GCP-associated genes, the aberrant tissue stem cells present at this time are not able to completely maintain high levels of these genes in the presence of bFGF (Figure 2-17, middle panel). Remarkably, cultured brain tumor stem cells upregulated both gene signatures and maintained them at high levels even in the presence of a differentiating stimulus expected to down-regulate Shh signaling and induce cellular differentiation (Figure 2-17, right panel).

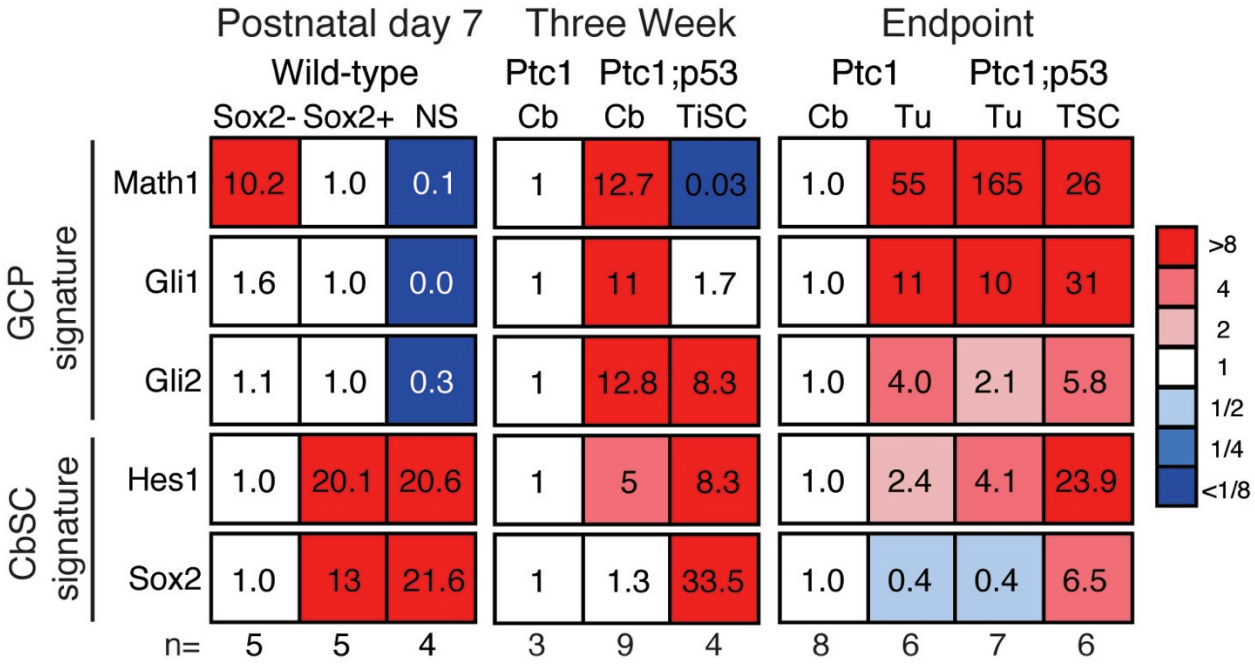

Figure 2-17 | Ptc1;p53 brain tumor stem cells highly express both GCP and CbSC gene signatures
qRT-PCR analyses for GCP and CSC gene signatures in the indicated genotypes and tissues show intact bFGF-dependent down-regulation of GCP signature in wild-type and pre-malignant tissue stem cells, but not brain tumor stem cells.

Give that neither Ptc1 pre-malignant cerebella nor full-blown Ptc1medulloblastoma are able to reproducibly self-renew or even be propagated *in vitro* in the presence of bFGF, we posited that *p53* deficiency must be vital for permitting the aberrant persistence and expansion of both pre-malignant tissue stem cells and brain tumor stem cells. While p53 has been shown to modulate self-renewal and differentiation in normal neural stem and progenitor cells as well as in tumor stem cell through both Shh-dependent and independent mechanisms[57,94], its role in the maintenance of cerebellar stem cell maintenance of self-renewal has yet to be directly investigated.

To approach this question, we compared p53 pathway activities between the CbSC and GCP populations of the p7 cerebella of *Sox2-GFP* reporter animals (Figure 2-18a). While we did not observe differences in p53 mRNA levels between these two compartments, the expression of p21, a cell cycle inhibitor and well-established p53 target gene, is consistent with decreased p53 pathway activity within the cerebellar stem cell compartment. Furthermore, we found concordant upregulation of another p53-responsive gene: the bHLH protein Id2, which has been recently shown to be directly bound and repressed by p53 within neural progenitors[104]. Moreover, Id2 has been reported to interact with and destabilize Math1 in the mouse Shh-medulloblastoma model[13]. Consistent with these findings suggesting that p53-dependent and Id2-mediated modulation of Math1 may facilitate differentiation of cerebellar stem cells down a granular cell lineage, we additionally observed a significant expansion of the Sox2+ cell population in Ptc1;p53;Sox2-GFP chimeric animals relative to wild-type Sox2-reporter animals (Figure 2-18b). Thus, cells deficient in p53 may be resistant to GCP-specification and more effectively colonize the cerebellar stem cell compartment.

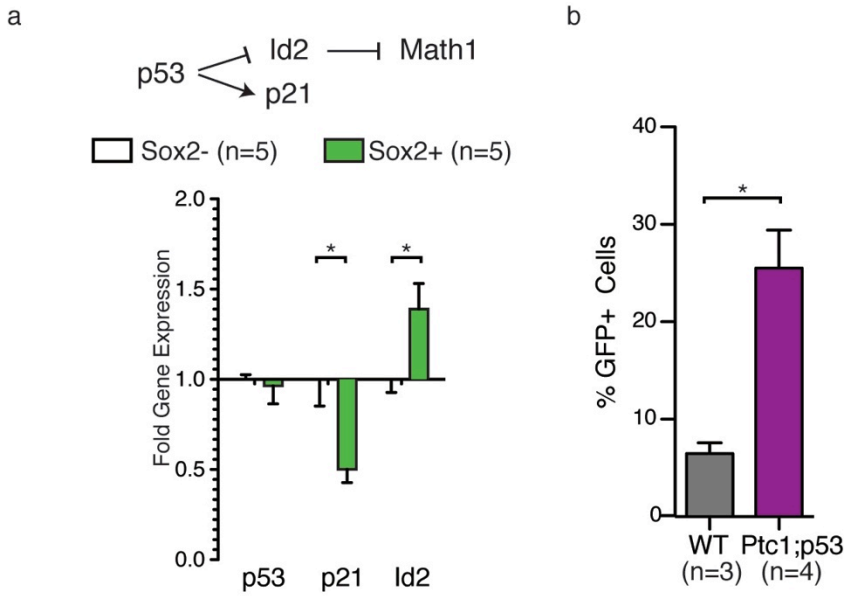

Figure 2-18 | Decreased p53 pathway activity may expand population of Sox2+ cerebellar cells
a, qPCR expression analyses for *p53* and two of its downstream effector genes, *Id2* and *p21*, show differences in p53 pathway activity between distinct cellular compartments of the wild-type cerebellum. b, Flow-cytometrc analyses of GFP-positivity in postnatal day 7 cerebellum shows expansion of Sox2+ population in the Ptc1;p53 but not wild-type genotype.

Discussion

Field cancerization was first described in epithelial malignancies in which tumor foci were observed to be surrounded by a field of overtly normal pre-malignant cells[92]. The resulting malignancies were typically multi-focal and frequently associated with *p53* mutation or loss[105]. Analogous to these observations and within the setting of chimeric organismal development, we have been able to ascribe a previously unappreciated role for *Ptc1* haploinsufficiency that allows mutant cells to competitively and preferentially seed a susceptible organ field. Specifically, we have observed that both Ptc1 and Ptc1;p53 mutant cells are non-randomly targeted to developmental regions where Shh is known to play a key role. Contrary to the classic two-hit tumor suppressor model where the first-hit is considered largely inconsequential to

tumorigenesis[106], our findings ascribe a much more ominous effect of tumor suppressor heterozygosity in these tumors. However, consistent with the two-hit model and definitively separating pre-malignant seeding from outright transformation, progression to full-blown malignancy within these tumors still requires the occurrence of *Ptc1* loss-of-heterozygosity. As in field cancerization, we observed synergistic interactions between *Ptc1* and *p53* resulting in a diffuse tumor phenotype as well as increased tumor kinetics and penetrance that are suggestive of polyclonal disease. Thus, early *Ptc1* and *p53* mutations collaborate within development in a non-random manner to pre-dispose an animal to cerebellar tumors, a phenomenon that we are terming developmental field cancerization (Figure 2-19).

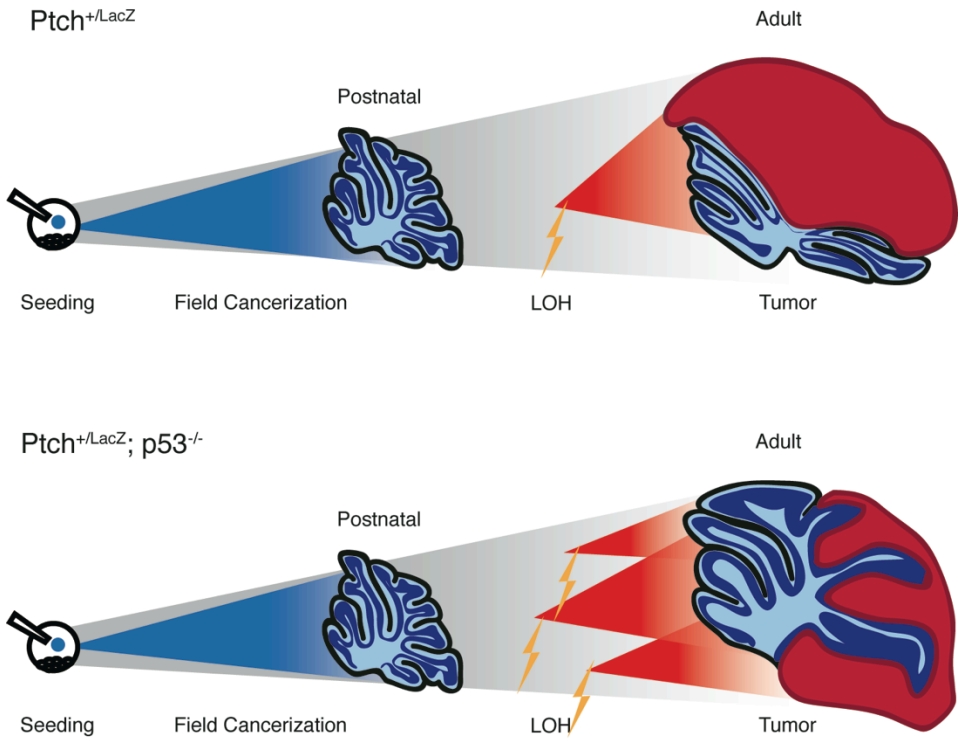

Figure 2-19 | Developmental field cancerization pre-disposes to malignant transformation through preferential seeding of the susceptible organ
Cartoon depicting the seeding of the cerebellar field with pre-malignant cells and the occurrence of *Ptc1* LOH leading to focal (Ptc1) and multi-focal, diffuse tumors (Ptc1;p53) with self-renewing and disseminated properties.

Surprisingly, we found that *p53* deficiency in the context of *Ptc1* mutation not only dramatically increases the rapidity and incidence of medulloblastoma formation but also fundamentally changes the properties of the resulting tumor. While it has been shown that *ex vivo* induction of oncogenic changes in isolated self-renewing cerebellar stem cells can lead to tumor-initiating cells[68-70], we report here the *in situ* transformation of an endogenous tissue stem cell specific to the Ptc1;p53 cerebellum, representing a bona fide *in vivo* mechanism leading to malignancy. Through further molecular characterization of and transplantation assays involving this unique subpopulation, we found Ptc1;p53 medulloblastomas to be heterogeneous tumors with heretofore unappreciated stem-like and metastatic qualities, and defined *Ptc1* LOH as the genetic switch allowing a pre-malignant tissue stem cell to become a tumor-initiating cell. Additionally, the utilization of a novel approach for marking and prospectively isolating these self-renewing cerebellar stem cells has allowed us to appreciate key differences in gene expression between mutant and wild-type cerebellar stem cells as well as to suggest a potential mechanism by which deficiency in *p53* allows for the prolonged persistence of these cerebellar stem cells.

Thus, while Ptc1 and Ptc1;p53 tumors may have been considered largely identical and interchangeable, we describe here within the Ptc1;p53 animals the identification of a unique sub-population of clonogenic brain tumor stem cells that surprisingly possess properties of both cerebellar stem and granule progenitor cell lineages. This population may function as yet another putative cell of origin and drive the more malignant and aggressive disease progression (Figure 2-20). Finally, while disseminated tumor growth has recently been proposed to arise from the stochastic accumulation of mutations within rare cells of the primary tumor[87], our work suggests that developmental field cancerization within the tissue stem cell compartment may not only

convey many of the properties required for metastatic growth but also dramatically amplify the occurrence of these 'rare' cells within the primary tumor. Elucidating the genetic pathways responsible for stem cell field cancerization, shown here to presage aggressive cancers, may be an important means of identifying mechanisms for therapeutic intervention of treatment-refractory tumors.

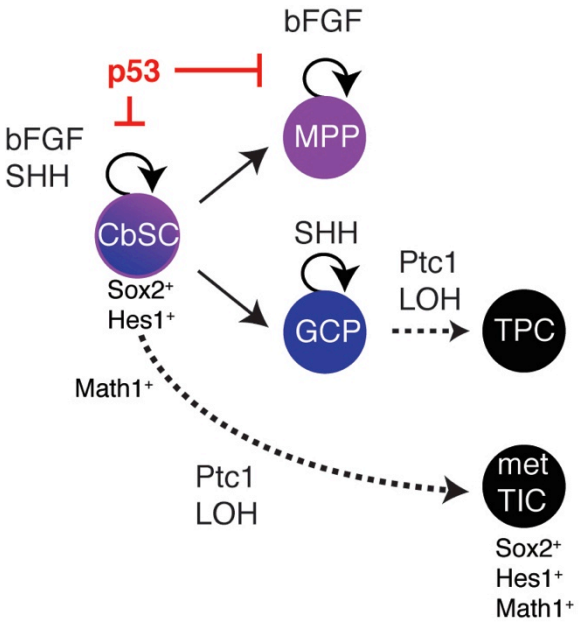

Figure 2-20 | Ptc1 and p53 mutations interactions lead to an alternate, stem-like cell-of-origin Model for polyclonal tumor initiation in Ptc1;p53 medulloblastomas derived from expansion of an early multi-potent cerebellar stem cell. CbSC cerebellar stem cell; GCP granule cell progenitor; MPP multi-potent progenitor; TIC tumor initiating cell; TPC tumor propagating cell.

Methods

ES cell line derivation and culture

Morula stage embryos were harvested at 2.5 dpc from superovulated females mated to appropriate males, and cultured in potassium simplex optimized media with amino acids (KSOM-AA) for two days[107]. The zona pellucida was removed from unhatched blastocysts with Acid Tyrode's solution (Sigma-Aldrich T1788). Each blastocyst was plated in one well of a 96 well plate on irradiated mouse embryonic fibroblast (MEF) feeders, and cultured in ES cell media containing 1000U/ml LIF (Millipore) and 50 uM MEK1 inhibitor (PD98059, Cell Signaling) for five days. Attached blastocysts were dissociated with trypsin and replated on MEFs in single wells of a 96 well plate. Wells containing colonies with ES cell morphology were expanded, genotyped, and cryopreserved. ES lines were function tested for chimera formation and germline potency. New lines characterized in this work include: $Ptc1^{LacZ/+}$; $p53^{null/null}$ (n=3), $Ptc1^{LacZ/+}$(n=2), $p53^{null/null}$ (n=1), *wt-lacZ Z/EG* (n=1). *EF1a-H2BmCherry* marked cells were generated by homologous recombination of the transgene adjacent to the *Collagen1a* locus to facilitate ubiquitous expression.

Production of mouse chimeras and animal husbandry

Chimeric mice were generated by injecting 5-10 ES cells into blastocyst stage embryos derived from superovulated females from CD-1 (Charles River) crosses. Embryos were isolated at day 2.5 pc, cultured in KSOM-AA overnight, and injected as expanded blastocysts prior to transfer to day 2.5 pc pseudopregnant CD-1 females. The $Ptc1^{LacZ}$ allele was derived from line $Ptc1^{tm1Mps/J8}$ (Jackson Labs, stock number 003081); the *p53* allele was derived from line 129-$Trp53^{tm1Tyj/J31}$ (Jackson Labs, stock number 002080); the *wt-lacZ* allele was derived from the Z/EG line[102].

Tissue from *Sox2-GFP* reporter mice were obtained from the Hochedlinger lab[98,99]. Mice were bred and maintained on a 129/B6 mixed background in pathogen-free conditions at the Children's Hospital Boston. All procedures were performed with Institutional Animal Care and Use Committee (IACUC) approval.

Genotyping, expression analysis, and copy number analysis by quantitative-PCR

Genomic DNA and mRNA for qPCR were isolated from liver (median lobe), cerebellum, tumor, and "midbrain" (comprising basal forebrain, thalamus, hypothalamus and the midbrain proper), and individual cultured neurospheres. Genomic DNA was prepared using standard isopropanol precipitation protocol. mRNA isolation was achieved using the Qiagen RNA Miniprep column preparations. Analyses were performed on a StepOnePlus machine (ABI).

neo- To determine relative contribution of ES and host cells in chimeric animals, frequency of the neomycin resistance (*neo*) gene was compared to the housekeeping gene *Gapdh* by qPCR using the delta-delta CT method. *Gapdh* copy number was chosen as a control as it has been shown not to vary in *p53* null tumors[108]. The $Ptc1^{LacZ}$ and $p53^{null}$ alleles each contain a single copy of *neo*. Genomic tail DNA from $Ptc1^{+/+}$; $p53^{+/+}$ (0 neo copies), $Ptc1^{LacZ/+}$; $p53^{+/+}$ (1 neo copy), $Ptc1^{LacZ/+}$; $p53^{null/+}$ (2 neo copies) and $Ptc1^{LacZ/+}$; $p53^{null/null}$ (3 neo copies) mice were used to create a standard curve for *neo* copy number.

Ptc1 wild-type- Frequency of the *Ptc1* wild-type (WT) allele was compared to *Gapdh* by qPCR to detect loss of heterozygosity (LOH) events at the *Ptc1* locus. Genomic DNA from $Ptc1^{LacZ/LacZ}$

(0 WT copies), *Ptc1*$^{LacZ/+}$ (1 WT copy), and *Ptc1*$^{+/+}$ (2 WT copies) DNA isolated from ES cell lines were used to create a standard curve for copy number. Primers used include:

Gapdh: 5'-ATAACGGCGGTTCATTCATT-3', 5'-GGGCTTCTTTCTTTACTTTCG-3'

neo: 5'-GGATCTCCTGTCATCTCACCT-3', 5'-ATCATCCTGATCGACAAGACC-3'

Ptc1: 5'-CTGCGGCAAGTTTTTGGTTG-3', 5'-AGGGCTTCTCGTTGGCTACAAG-3'

Quantitative Expression Analyses – RNA isolated as described above and converted to cDNA using the ABI high-capacity cDNA reverse transcription kit. Expression values were calculated by delta-delta CT method and normalized to *Gapdh*. Primers used are as follow:

Gapdh: 5'- GTTGTCTCCTGCGACTTCA -3', 5'- TGGTCCAGGGTTTCTTACTC -3'

Gli1: 5'- TTCAAGGCCCAATACATGCT -3', 5'- GCGTCTTGAGGTTTTCAAGG -3'

Gli2: 5'- GGTCTCTTGGAGGACAGCAG -3', 5'- TCTCATGTCAATCGGCAAAG -3'

Hes1: 5'- ATGCCGGGAGCTATCTTTCT -3', 5'- ACACCGGACAAACCAAAGAC -3'

Id2: 5'- CCGCTGACCACCCTGAAC -3', 5'- CCGCTGACCACCCTGAAC -3'

Math1: 5'- ACAGGTCCTTCTGTGCCATC -3', 5'- GCTTCCTCTGGGGGTTACTC -3'

p21: 5'- ATGTCCAATCCTGGTGATGT -3', 5'- TGCAGCAGGGCAGAGGAAGT -3'

Sox2: 5'- CCGAGGAGGAGAGCGCCTGT -3', 5'- GCTCGAGACGGGCGAAGTGC -3'

p53: 5'- TGAAACGCCGACCTATCCTTA -3', 5'- GGGCACAAACACGAACCTCAAA -3'

Histology and Xgal staining

For H&E staining, paraformaldehyde-fixed tissues were dehydrated with an ethanol series, xylene, then embedded with Paraplast. 5 micron sections were dewaxed, rehydrated and stained

with hematoxylin-eosin. For Xgal staining, tissues were fixed in 2% paraformaldehyde, 0.2% gluteraldehyde for 1 hour at room temperature, washed in PBS and stained for 16-18 hours at 20°C (Ptc1^{lacZ} lines) or 6-8 hours at 25°C (Z/EG lines) in Xgal buffer (1xPBS, 2mM MgCl2, 0.1% Sodium deoxycholate, 0.2% Nonidet P-40, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-Gal). Stained tissue was washed in rinse buffer (1xPBS, 2mM MgCl2, 0.1% Sodium deoxycholate, 0.2% Nonidet P-40) and postfixed in 4% paraformaldehyde overnight at 4°C.

Neurosphere culture, Low-density Neurosphere formation and Clonogenicity assays

Bulk cultures- Tissues were isolated, minced, and triturated in Accutase (Millipore) to obtain a single cell suspension. After centrifuging, cells were grown in neurosphere (NS) media consisting of DMEM/F12 supplemented with 20ng/ml of bFGF, 20ng/ml of EGF, B27 supplement, and penicillin/streptomycin[109]. Bulk neurosphere cultures were passaged by disaggregation with Accutase and maintained in NS media.

Clonal analyses- To quantify self-renewal, neurospheres were dissociated into single cells using Accutase, counted, and plated at 1500 cells/ml in NS media in six-well replicates. Clonal neurospheres were counted after two weeks. For the 96-well clonogenicity assays, limiting dilutions of cells were plated in replicate wells in a 96 well plate in 100µl of NS media per well. Wells were scored for the presence of neurospheres and the frequency of positive wells used to calculate the frequency of self-renewing cells using the Poisson distribution[110].

Immunostaining

Neurospheres were fixed with 4% PFA and 2% sucrose for 20 minutes, permeabilized with 0.4% Triton-X 100 for 1hr, blocked for 30 minutes with 5% normal goat serum, 5% normal donkey serum, 0.02% Tween-20 in Tris Buffered Saline, and incubated overnight at 4ºC in the presence of primary antibodies (Nestin, AbCam, 1:100; Sox2, R&D Systems, 1:1000). Washes used 0.02% Tween-20 in Tris Buffered Saline, and secondary antibodies used were Cy2 donkey anti-rabbit and Cy3 donkey anti-mouse (Jackson Immunoresearch, 1:500). Visualization was performed on a Zeiss LSM510 META 2-photon confocal microscope. For growth factor withdrawal analysis, cells were cultured adherently on poly-L-lysine coated wells (Sigma) for one week in the presence or absence of growth factors and stained as above. ImageJ was used for quantification of cellular staining intensity.

Flow cytometry

Tissues were dissociated into single-cell suspensions as described above for neurosphere culture. Cells were resuspended in PBS + 2mM EDTA + 0.5% BSA and incubated for 30 minutes with PE-conjugated CD15 (R&D Systems), PE-conjugated CD133/Prominin (Miltenyi), and Alexa647-conjugated CD184 antibodies (eBioscience). Samples were subsequently run on a BD LSRII system gating on live cells through excluding debris and dead cells using FSC/SSC parameters and 7AAD staining (BD Biosciences). Gates for marker positivity were determined through staining of samples with anti-mouse isotype controls (Miltenyi Biotec). Analyses were performed and graphs generated using FlowJo (Tree Star). Cell sorting experiments were done in conjunction with the HSCI/Joslin Flow Cytometry Core on the BD FACSAria.

Intracerebellar transplantation assays

NCr nude (*CrTac:NCr-Foxn1^{nu}*) mice (4-6 week old) were anesthetized with ketamine/xylazine and placed into a stereotaxic frame (Stoelting). A 1cm incision was made in the scalp and a Dremel drill was used to bore a hole approximately 1mm lateral and 3mm posterior to lambda. Primary tissues were dissociated into single cell suspensions as described above. $5x10^4$ cells in 2ul of PBS were drawn into a pulled glass needle and delivered into the cerebellum 3mm below the surface using a PicoPump (World Precision Instruments). The incision was closed using EZ Clips (Stoelting) and the animals monitored daily for the development of neurological symptoms.

Statistical analyses

All graphs were generated and statistics performed using Prism 5.0 (Graphpad Software). Statistical analyses for self-renewal and Ptc1 wild-type allele quantification were performed using the Kruskal-Wallis Test with Dunnett's Multiple Comparison Test to determine statistically significant differences between different genotypes. Comparison of CD184 surface expression was performed using two-way ANOVA with Bonferroni post-tests for the different genotypes. CD15 surface expression was compared using Student's *t*-test. Comparisons between survival curves were performed using the Mantel-Cox log-rank test. All experiments with error bars are represented as the mean ± standard error.

Author Contributions

Initial Ptc1 and Ptc1;p53 ES lines were derived and maintained by CDK. JB was responsible for generation of all chimeric animals and animal husbandry. Quantitative genomic DNA qPCR assays for ES contribution in chimeric animal tissue were performed by CDK, CYL, and JB; histology and XGal staining was performed by JB. Isolation, generation, and subsequent characterization of neurospheres and self-renewing cells were performed by CYL. Intra-cerebellar transplantation assays were performed by CYL and AP. Quantitative mRNA expression analyses from isolated tissues and cells were performed by CYL.

Tissue from wild-type Sox2-reporter animals were provided by AS and KA of the Hochedlinger Lab; flow cytometric marker analyses and fractionation as well as subsequent expression analyses were performed by CYL. Sox2-reporter plasmid constructs were obtained from the Hochedlinger Lab; recombinant modification of the Sox2-GFP plasmids and subsequent generation of Sox2-reporter ES lines in v6.5 and Ptc1;p53 genetic backgrounds were performed and maintained by CYL. LJG helped design experiments, interpreted data, and edited the manuscript.

Chapter 3

Kcnq1ot1 non-coding RNA is involved in medulloblastoma brain tumor stem cell self-renewal

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Introduction

Treatment for medulloblastoma, and indeed cancers in general, is often complicated by tumor heterogeneity. Prior to the molecular characterization and understanding of the many pathways and genes involved in tumor formation, cancer therapy has relied upon indiscriminate treatment modalities that preferentially target rapidly dividing cell populations. Such therapies remain the current standard of care for medulloblastoma patients[111]. Molecular genetics has led to the identification of four medulloblastoma subtypes characterized by deregulation of distinct, often non-overlapping gene programs that respond differentially to conventional therapy[2], driving the development of targeted therapies[42-44]. Indeed, continued advances in our molecular characterization of these medulloblastoma have led to the recent realization that intra-tumor heterogeneity represents yet another important aspect of tumor biology meriting careful investigation and consideration in the development of new treatment modalities.

Within some medulloblastoma, a further definable subset of cells with stem-like character has been identified that are generally marked by neural stem cell markers, associated with poor disease prognosis, and able to give rise to robust tumors. For example, fractionation of primary tumors for the cell surface marker CD133 has demonstrated dramatic enrichment for tumor-initiating capabilities in the CD133-positive fraction in human tumor samples[58]. The observations that no obvious karyotypic differences exist between CD133-positive and negative fractions[77] as well as that fractionated CD133-negative cells demonstrate tri-lineage potential and the ability to regenerate CD133 marker expression are suggestive of a role for epigenetic regulation in the maintenance of stem-like properties in these cells[97]. Consistent with this concept, genetic analyses of human medulloblastoma samples have shown that a significant

fraction of tumors carries mutations in histone modifying genes and aberrancies in DNA methylation of key developmental and signaling pathways. Furthermore, Bmi1, a member of the polycomb repressive complex 1 (PRC1) involved in epigenetic gene silencing[33,34,69,70,72,83,84,98,99,112,113], has been found to regulate normal cerebellar and tumor stem cell self-renewal and to be critical for tumor-initiation in mouse models. Interestingly, it has been shown that Bmi1 interacts with DNA methyltransferase 1 (Dnmt1)[80], an enzyme required for maintenance of genomic imprinting and involved in gene silencing[74], suggesting that significant functional interplay may also occur between these two epigenetic mechanisms in cerebellar development. Furthermore, recent observations that polycomb targets in embryonic stem cells are frequently found to be hypermethylated in a variety of malignancies[114,115] suggest that the epigenetic states of stem and progenitor cells may be particularly important in pre-disposition to tumorigenesis.

We had previously identified a unique sub-compartment of tumor-initiating cells within Ptc1;p53 medulloblastoma tumors that possesses stem-like properties and the ability to give rise to aggressive, disseminated secondary tumors upon transplantation. Interestingly, these cells were found to express marker genes for both cerebellar stem cells as well as granular cell progenitors. Furthermore, we identified genetic loss-of-heterozygosity at the *Ptc1* locus as necessary for tumorigenesis in both Ptc1 and Ptc1;p53 animals. Given that we observed *Ptc1* LOH in the bulk tumor as well as in the self-renewing tumor-initiating stem cell compartment, we now sought to ask what additional epigenetic changes at the level of DNA methylation may exist within the BTSC population that distinguish it from the rest of the tumor. Here we show that Ptc1;p53 brain tumor stem cells differ from the bulk tumor tissue in promoter methylation status of the

imprinted genes *Kcnq1ot1* and *Cdkn1c*. Through mRNA expression analysis and RNA-FISH, we observe aberrant bi-allelic expression of *Kcnq1ot1* in the BTSC compartment. Finally, lentiviral-mediated knockdown of Kcnq1ot1 leads to decreased tumor stem cell self-renewal, suggesting a role for the long non-coding RNA in BTSC maintenance.

Results

Loss of imprinting has been observed in a number of malignancies and developmental diseases[116]. Moreover, global loss of imprinting has been shown to pre-dispose to adult tumorigenesis in mice[117]. In order to begin characterization of differences in DNA methylation between Ptc1;p53 brain tumor stem cells (n=5) and primary tumor samples (n=5), we queried the methylation status of 78 imprinted genes through bisulfite-based mass spectrometry methylation assays that allowed us to measure the methylation state of individual CpGs in a high-throughput screen[118]. We observed that 10 of the 78 imprinted gene promoters examined demonstrated significant and reproducible differences in DNA methylation between the primary tumor and BTSC samples (data not shown, summarized in Figure 3-1), consistent with a role for DNA methylation in distinguishing between tumor and BTSC.

Gene	Exp Allele	Chr.	Meth. Change	Average Meth.
Gatm	M	2	Gain	42%
Asb4	M	6	Gain	70%
Ndn	P	7	Gain	58%
Magel2	P	7	Gain	64%
Cdkn1c	M	7	Gain	72%
Kcnq1ot1	P	7	Loss	15%
Rasgrf1	P	9	Gain	59%
Plagl1	P	10	Loss	23%
Dlk1	P	12	Gain	47%
Dio3	P	12	Gain	43%

Figure 3-1 | BTSC-specific changes in DNA methylation. Table cataloguing the changes in promoter methylation of imprinted gene loci identified between Ptc1;p53 brain tumor stem cells and Ptc1;p53 primary tumor samples, the allele from which the genes are normally expressed, as well as the directionality of the methylation change in BTSC.

We were immediately intrigued by a set of coordinated changes identified in the BTSCs: simultaneous hypermethylation at *Kcnq1ot1* (*Lit1*) and hypomethylation at *Cdkn1c* (*p57*) (Figure 3-2a) that are consistent with loss-of-imprinting occurring within the BTSC at these loci. Mean methylation was decreased to less than 20% at the *Kcnq1ot1* locus and dramatically increased to nearly 80% at the *Cdkn1c* locus (Figure 3-2b). The cyclin-dependent kinase inhibitor p57 plays a well-established roles in regulating cell cycle exit[119] and its regulation is known to be involved in maintenance of both pancreatic as well as intestinal crypt progenitor cell populations[120,121]. In zebrafish retina, Shh-regulated activation of Cdkn1c has been shown to necessary and sufficient for cell cycle exit of retinal progenitor cells[122]. Within medulloblastoma, high levels of Cdkn1c are associated with Notch pathway activation and observed in the Wnt and Shh subtypes of medulloblastomas[82].

The *Cdkn1c* gene locus exists as part of an imprinting cluster that has been shown to be regulated by the expression of the 90+kb long, non-coding, and paternally expressed RNA[112], Kcnq1ot1 (Figure 3-3a). Although the exact mechanism by which Kcnq1ot1 modulates *Cdkn1c* promoter methylation remains an area of active research, it has been observed to directly bind to and interact with Dnmt1 in mediating silencing of *Cdkn1c* among other genes[123]. Furthermore, hypomethylation in the imprint control region of *Kcnq1ot1*, associated with silencing of *Cdkn1c* expression, is often observed in Beckwith-Wiedemann syndrome, an overgrowth condition with predisposition to malignancy[124]. As such, it is conceivable that loss of imprinting occurring in the *Kcnq1ot1*-imprinted gene cluster in the TSC may allow the cells to aberrantly silence *Cdkn1c* expression via an imprinted gene network in order to maintain self-renewal potential and resist differentiation (Figure 3-3b).

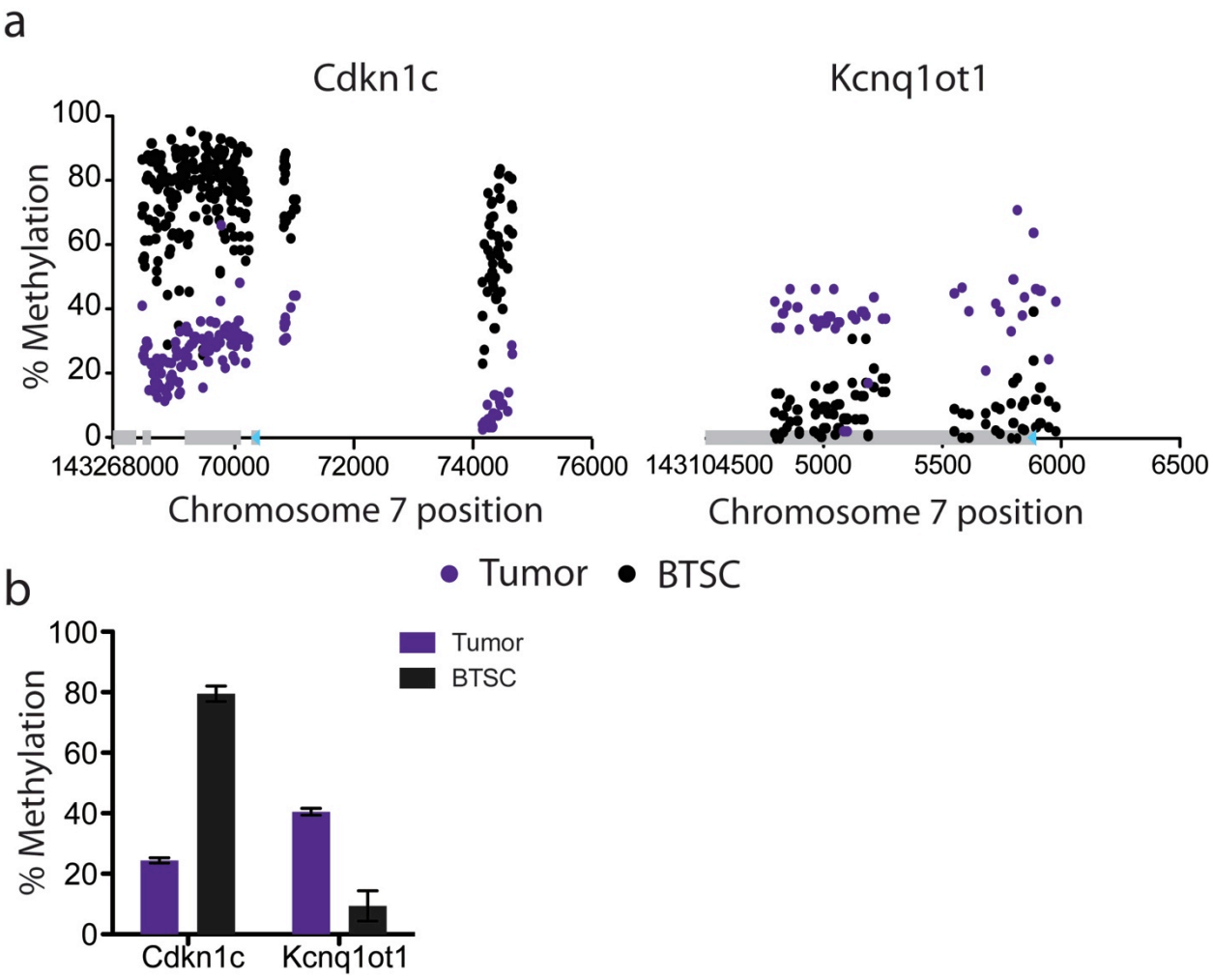

Figure 3-2 | Coordinate changes in promoter methylation for *Cdkn1c* and *Kcnq1ot1* are observed in BTSC

a, Examination of the MassArray data demonstrates complete hypomethylation at the *Kcnq1ot1* gene locus coupled with hypermethylation at the *Cdkn1c* gene locus, quantified in (b). Transcriptional start sites are denoted by blue arrowheads and gray boxes indicate the exons of the resulting transcript.

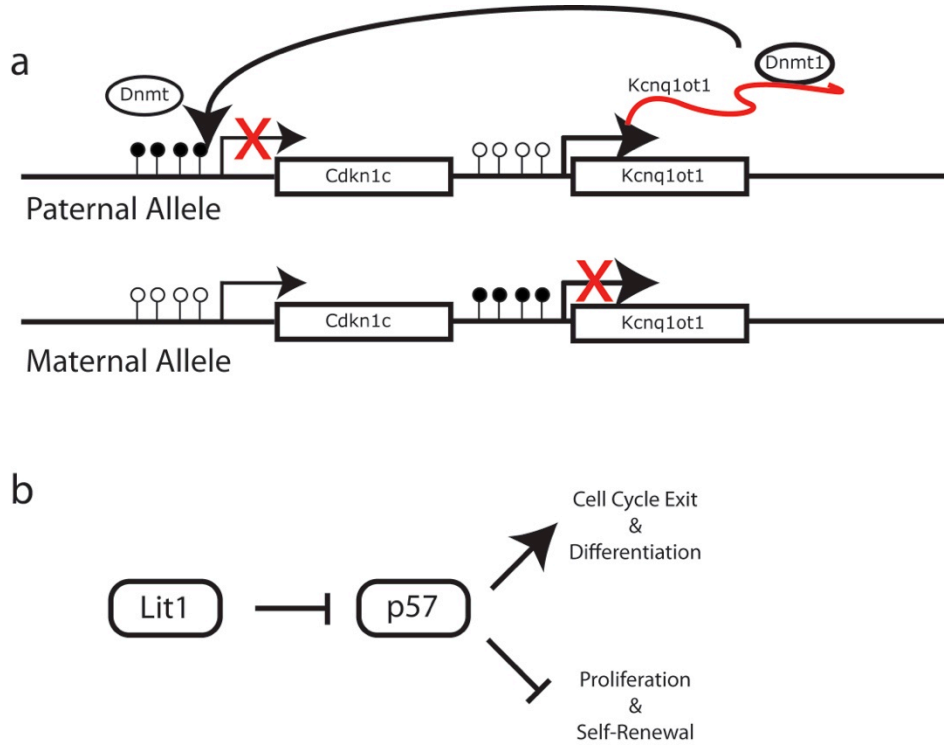

Figure 3-3 | Kcnq1ot1 modulates transcription of Cdkn1c
a, Schematic illustrating the relationships between promoter methylation states of *Kcnq1ot1* and *Cdkn1c* normally. Expression of the Kcnq1ot1 RNA and interaction with Dnmt1 has been shown to be required for silencing of the *Cdkn1c* promoter. b, Kcnq1ot1 suppression of *Cdkn1c* expression is a potential mechanism in the maintenance of BTSC.

In order to see whether the observed differences in methylation are reflected at the level of gene expression, we examined mRNA levels for Cdkn1c and Kcnq1ot1 in primary tumor tissue as well as in BTSCs. Consistent with the promoter methylation status described earlier, we found Kcnq1ot1 expression increased by approximately 2-fold and Cdkn1c decreased nearly 10-fold in the BTSC relative to primary tumor tissue samples (Figure 3-4a). Given the changes in gene expression, we hypothesized that loss-of-imprinting had occurred within the BTSC lines involving bi-allelic activation of the Kcnq1ot1 RNA expression. Indeed, we observed bi-focal punctate localization of the Kcnq1ot1 RNA within the majority of BTSC examined but not in primary tumor cells (Figure 3-4b, c). These findings are consistent with our previous observation that approximately 1 in 3 brain tumor stem cells but only 1 in 500 primary tumor cells are

capable of *in vitro* self-renewal (Figure 2-13a, data not shown). Thus, methylations changes at the *Kcnq1ot1*/*Cdkn1c* gene loci observed in the BTSC are associated with coordinate changes in RNA expression levels and enriched for in a purified population of self-renewing, tumor-initiating stem cells.

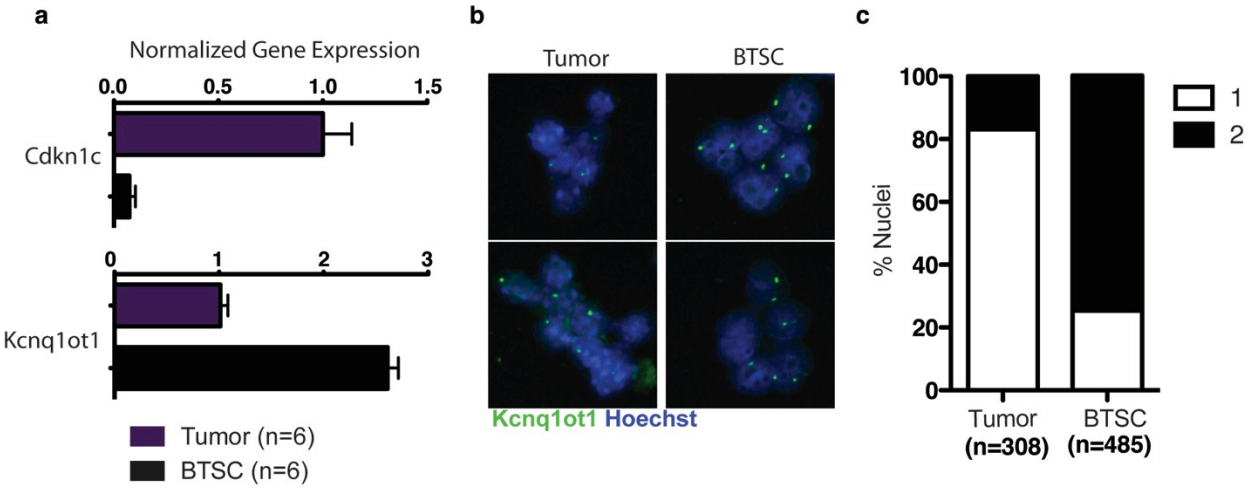

Figure 3-4 | Bi-allelic expression of *Kcnq1ot1* and silencing of *Cdkn1c* is specific to BTSC
a, qPCR quantification of Cdkn1c and Kcnq1ot1 transcripts in Ptc1;p53 tumor and BTSC samples shows reciprocal changes in RNA level for the two genes. b, Representative image from RNA-FISH against Kcnq1ot1 RNA in tumor and BTSC samples, quantified in (c).

Work in breast cancer cell lines has shown that transcriptional silencing of *Cdkn1c* can occur through multiple epigenetic mechanisms[125]. In order to parse the mechanisms by which silencing of the *Cdkn1c* promoter occurs, BTSC lines (n=6) were treated with the DNA methyltransferase inhibitor 5-Aza-deoxycytidine (5Aza) singly or in conjunction with the HDAC inhibitor Trichostatin A (TSA). We found that 5Aza treatment alone was able to reactivate Cdkn1c mRNA expression by more than 10-fold, while TSA treatment had no additive effect on transcription (Figure 3-5a). In addition, treatment of BTSC with 5Aza was found to abrogate self-renewal of tumor neurospheres in nanomolar concentrations (Figure 3-5b). Finally, we observed significant demethylation of the *Cdkn1c* gene locus in all treated BTSC lines by bisulfite-based mass spectrometry methylation assays (Figure 3-5c). Taken together, these

findings suggest that DNA methylation may be an important means of *Cdkn1c* regulation in BTSCs contributing to the maintenance of self-renewal.

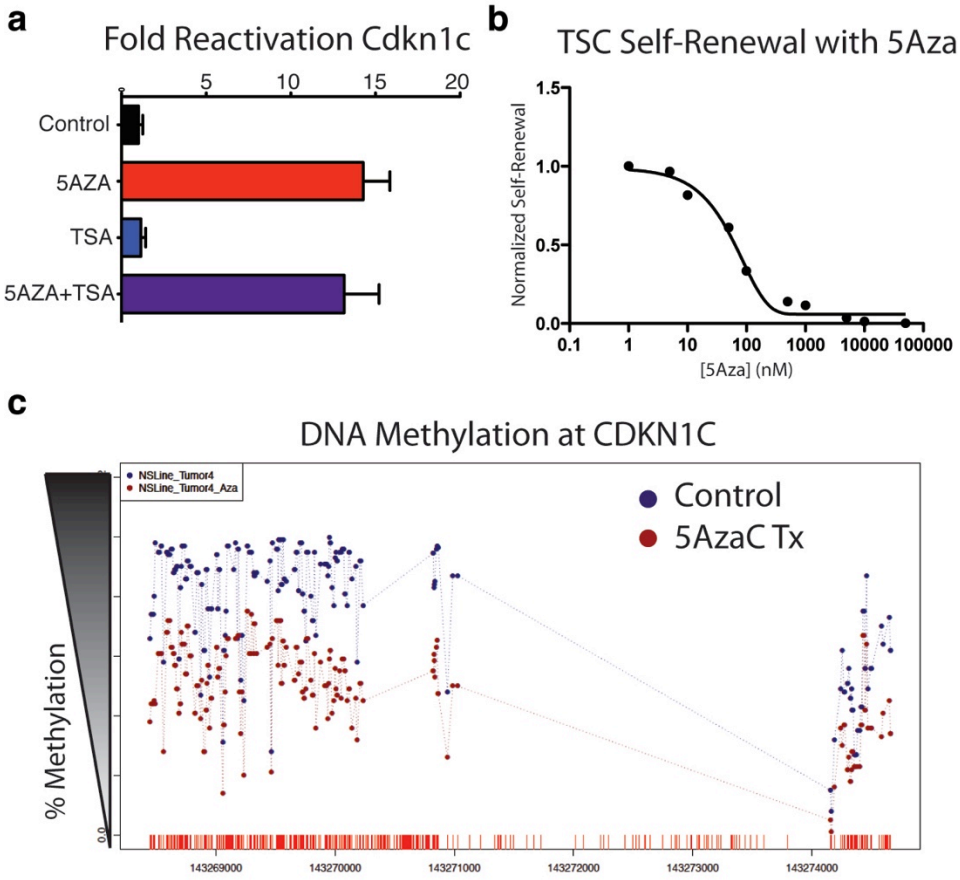

Figure 3-5 | Pharmacologic inhibition of DNA methylation in BTSC reduces self-renewal
a, Inhibition of DNA methylation, but not histone deacetylation, reactivates *Cdkn1c* expression and leads to demethylation of the *Cdkn1c* promoter, shown in (c). b, 5Aza treatment demonstrates dose-dependent decreases in self-renewal activity.

Having characterized these molecular differences between the Ptc1;p53 BTSC and bulk compartments, we now sought to functionally investigate the role of *Kcnq1ot1* expression in BTSC through lentiviral-mediated shRNA knockdown against Kcnq1ot1. Brain tumor stem cells were infected with empty vector, control hairpin (CTL-SH), or a hairpin targeted against the Kcnq1ot1 transcript (shLit1). Following selection, infected BTSC were plated at clonal density

to allow quantification of *in vitro* self-renewing capability. We observed a 75% decrease in self-renewing neurospheres numbers as well as a 50% reduction in neurospheres size for shLit1-infected cells compared to those with empty and control vectors (Figure 3-6a, b). Finally, the shLit1-treated BTSCs showed a nearly 10-fold reactivation of *Cdkn1c* expression (Figure 3-6c), comparable to the level of *Cdkn1c* expression we observed following pharmacologic inhibition of DNA methylation. While these observations have only been found in a single hairpin, these data potentially support a role for the long non-coding RNA Kcnq1ot1 in BTSC self-renewal through the modulation of *Cdkn1c* expression.

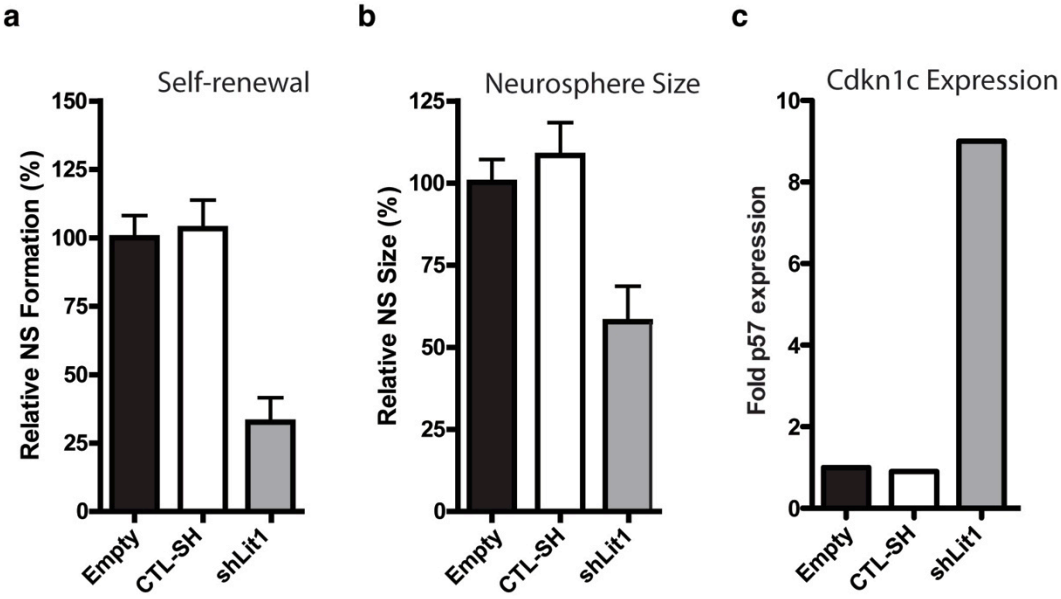

Figure 3-6 | Kcnq1ot1 knockdown in BTSC leads to decreased self-renewal and proliferation
a, Quantification from low-density self-renewal assay following lentiviral infection and knockdown. The average size of neurospheres observed was measured and shown in (b). c, Reactivation of *Cdkn1c* expression was observed in one of the hairpins utilized.

Discussion

The key to creating novel and effective therapeutics for difficult to treat tumors revolves around developing an understanding of the molecules and pathways that make a particular tumor aggressive and refractory to therapy. Work by others in the field have shown that rare sub-populations of stem-like cells may drive the most malignant disease and that clonal expansion of rare mutants within primary tumors may be underlying metastatic medulloblastoma[87]. As such, the intrinsic heterogeneity of the tumor and the lack of a good marker for prospectively isolating these cells have hampered their close investigation. Through our previous work, we have identified a unique population of brain tumor stem cells within Ptc1;p53 medulloblastoma and generated a highly purified and bioactive population through *in vitro* culture that represents an ideal system by which to characterize the differences separating the BTSC sub-compartments and the primary tumor from which they are derived.

From a screen of 78 imprinted genes, we observed consistent and significant promoter methylation differences between the Ptc1;p53 primary tumors and the brain tumor stem cell lines, implying that distinct epigenetic states do exist between these two cell populations. In order to further characterize these observed differences, we focused on a set of coordinate changes between a known imprint control element, the long non-coding RNA, Kcnq1ot1, and one of its target genes, the cyclin-dependent kinase, Cdkn1c, for further study. Consistent with the occurrence of loss-of-imprinting at these loci in BTSC being functionally significant, we observed silencing of *Cdkn1c* expression concurrent with enrichment for bi-allelic expression of *Kcnq1ot1*, and reactivation of *Cdkn1c* expression as well as abrogation of self-renewal activity through inhibition of DNA methylation in the BTSC. These observations were further supported

by shRNA-mediated knockdown of Kcnq1ot1 associated with decreased self-renewal and proliferative capacity in the BTSC.

Interestingly, while it is well known that Cdkn1c plays a role in progenitor maintenance in other organ systems[120,126] and even within some populations of neural progenitors[122], its role in cerebellar development and differentiation, if any, is largely uncharacterized. Indeed, of the many cyclin dependent kinases, only p21 and p27 have been reported to be significantly expressed in the developing cerebellum[127]. Specifically, p27 has been shown to be involved in controlling GCP proliferation and in suppressing medulloblastoma formation in a mouse model[128]. Given that the Ptc1;p53 BTSCs express markers of both cerebellar stem cells and granule cell progenitors, we speculate that modulation and silencing of *Cdkn1c* expression may represent a novel mechanism by which these cells are able to resist normal cues for differentiation and aberrantly persist to form tumors with stem-like characteristics.

Finally, non-coding RNAs such as microRNAs have been widely implicated in maintaining neural stem and progenitor populations[129], and specifically in modulating Shh signaling in cerebellar progenitors as well as in driving malignant progression[130]. However, the role of long non-coding RNAs in modulating neural progenitor differentiation and maintenance is as yet undefined. To our knowledge, this work represents the first report to implicate the activity of a long non-coding RNA in modulating neural stem cell self-renewal. Thus, the careful characterization of the roles of Kcnq1ot1 and Cdkn1c within the context of malignant BTSCs as well as normal cerebellum merits further investigation and consideration.

Methods

Neurosphere culture, Low-density neurosphere formation and clonogenicity assays

293T culture- Human 293T cells were maintained adherently in DMEM supplemented with 10% Cosmic calf serum (Gibco). Passaging was carried out through trypsinization, trituration, and re-plating of cells.

Bulk cultures- Tissues were isolated, minced, and triturated in Accutase (Millipore) to obtain a single cell suspension. After centrifuging, cells were grown in neurosphere (NS) media consisting of DMEM/F12 supplemented with 20ng/ml of bFGF, 20ng/ml of EGF, B27 supplement, and penicillin/streptomycin[109]. Bulk neurosphere cultures were passaged by disaggregation with Accutase and maintained in NS media.

Clonal analyses- To quantify self-renewal, neurospheres were dissociated into single cells using Accutase, counted, and plated at 1500 cells/ml in NS media in six-well replicates. Clonal neurospheres were counted after two weeks. For the 96-well clonogenicity assays, limiting dilutions of cells were plated in replicate wells in a 96 well plate in 100µl of NS media per well. Wells were scored for the presence of neurospheres and the frequency of positive wells used to calculate the frequency of self-renewing cells using the Poisson distribution[110].

Generation of short hairpin RNAs

Briefly, short hairpin RNA sequences targeted against Kcnq1ot1 were generated using pSicoOligoMaker 1.5 software as well as the DSIR algorithm[131] (http://biodev.extra.cea.fr/DSIR/DSIR.html), and cloned into the pSicoR-puro vector, a modified

pSicoR vector that was originally a gift from Dr. Tyler Jacks (Whitehead Institute, MIT), allowing puromycin selection following infection via established protocol. Selected sequences were screened through the NCBI-BLAST software to minimize off-targeting and ligated DNA colonies were screened for hairpin insertion by a PCR assay and sequenced for validation. Validated clones were grown up and plasmid DNA was isolated via Midi-prep columns (Qiagen) for lentiviral transfections.

Lentivirus production and infection

293T cells were plated onto gelatinized 10cm tissue culture dishes at ~30% confluency the day prior to transfection. Calcium phosphate transfection was performed using 15ug of the pSicoR-Puro-shRNA vectors, 10ug PAX packaging vector, and 5ug VSV lentiviral vector in 10mL of fresh media. Sixteen hours following transfection, the 293T media was changed to NS media and viral supernatants harvested 48 hrs following transfection. To infect BTSC, ~10^6 dissociated cells were spun-down and re-suspended with 2ml of viral supernatant supplemented with 10ug/ml polybrene in 6-well plates. Plates were spun at 2000 rpm for 45 minutes. Cells were subsequently collected, spun down, and resuspended in fresh NSM. Puromycin selection was begun 24 hours post-infection and maintained for 2 days in order to enrich for infected cells.

Quantitative RNA expression analyses

Total RNA were prepared from primary tissue and neurospheres cultures using RNA Mini Spin columns (Qiagen) and converted to cDNA using the ABI high-capacity cDNA reverse transcription kit. Expression values were calculated by delta-deltaCt method and normalized to Gapdh. Primers were as follow:

Gapdh: 5'- GTTGTCTCCTGCGACTTCA -3', 5'- TGGTCCAGGGTTTCTTACTC -3'

Lit1: 5'- GGCAGGCAGGATTAACCAGAT -3',

5'- AGAACTATAATCAAGCAATCCAATCCTT -3'

p57: 5'- GAAGAAGTCGTTCGCATTGGC -3', 5'-CGAGGAGCAGGACGAGAATC -3'

Bisulfite-based mass spectrometry methylation assay

For methylation assays, genomic DNA from tissue and BTSC lines were isolated using standard isopropanol precipitation and purified by phenol-chloroform extraction. Quantitative methylation analyses were performed on the MassArray EpiTYPER platform (Sequenom, San Diego)[118]. Briefly, sodium bisulfite treated DNA is transcribed in vitro, subjected to base-specific cleavage, and analyzed by MALDI-TOF MS. PCR amplicons were designed to cover CpG islands and 5' promoter regions of selected genes.

RNA fluorescence *in situ* hybridization (RNA-FISH)

Briefly, single cell suspensions were created from BTSC cultures and primary tumor samples. Cells were fixed with 4% PFA, permeabilized with 0.5% Triton in DEPC-PBS, and plated onto glass slides. Probe hybridization and subsequent procedures were performed as described by Q. Wang (personal communication) and Mohammad *et al*[132]. Plasmid with the Lit1-probe was a generous gift from Dr. M. J. Higgins (Roswell Park Cancer Center, Buffalo, NY).

Author Contributions

Collection of cerebellar and tumor tissue and subsequent mRNA expression analyses were performed by CYL and GW. RNA FISH experiments were performed by GW. Generation and validation of lentiviral hairpins against Kcnq1ot1 and Cdkn1c was performed by GW in conjunction with CYL. Experiments involving pharmacologic inhibition of methylation and reactivation of Cdkn1c were performed by CYL. LJG helped design experiments, interpreted data, and edited the manuscript.

Bisulfite-based methylation analyses were performed by TJ and ME of Sequenome.

Chapter 4 Perspectives and Future Directions

"Down to their innate molecular core, cancer cells are hyperactive, survival-endowed, scrappy, fecund, inventive copies of ourselves."

-- Siddhartha Mukherjee[133]

Whether it occurs in adults or children, cancer is fundamentally a developmental disease that is marked by deregulation of critical developmental pathways[134]. As such, the characterization and study of processes critical for tumorigenesis and normal developed are intertwined. While the advent of high-throughput tools for analyzing the genetic landscape of large cohorts of tumors has allowed us to look ever deeper into tumors and identify the slightest of differences, the challenge now arises in distinguishing the meaningful driver mutations from those that are not and functionally characterizing those changes to understand the underlying tumor biology. In this, the investigation of pediatric malignancies such as medulloblastoma may prove particularly rewarding as they generally contain fewer genomic changes than adult malignancies[76] and thus may more clearly highlight the important pathways and processes involved in development and tumorigenesis.

It has been this sense of discovery and wonder in attempting to study and understand the delicate balance between normal development and progression to malignancy that has fascinated me the most in the last few years and that will continue to drive my work in the future. What follows is an overview of my ongoing efforts as well as some of my personal thoughts on the significance of my contributions while part of the Jackson-Grusby lab.

Insights from mouse medulloblastoma models into human disease

The use of mouse models has been integral in furthering our understanding of the developmental bases and cellular origins of medulloblastoma formation. Integrated genomic approaches have allowed the division of medulloblastomas into four different subgroups that differ in terms of age of incidence, tumor histology, gene expression, and prognoses. Interestingly, SHH-type tumors occur in a bimodal age distribution: primarily either before 3 years or after 15 years of age[2]. Furthermore, molecular characterization of these early and late occurring SHH-type tumors have shown them to be clinically, transcriptionally, and prognostically distinct[135] although the biological mechanisms driving the formation of the earlier tumor-type remain to be clearly elucidated. Given that the addition of a p53-mutation dramatically increases medulloblastoma incidence and accelerates tumor formation[34], comparisons of the Ptc1 and Ptc1;p53 mouse medulloblastoma models may provide some initial insight into these differences (Figure 4-1).

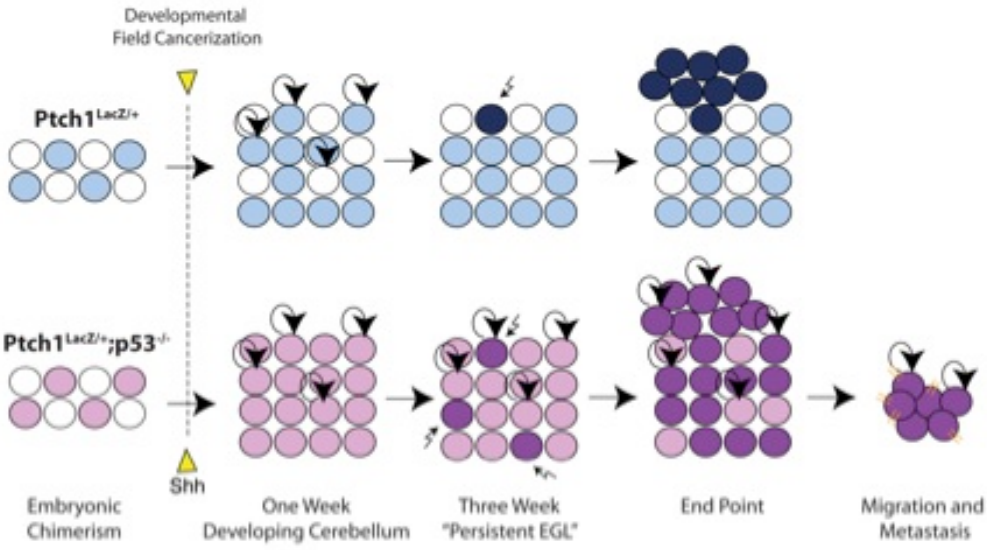

Figure 4-1 | Ptc1 and Ptc1;p53 medulloblastoma are developmentally and functionally distinct
Schematic illustrating the key differences distinguishing Ptc1 and Ptc1;p53 medulloblastoma. Namely, the complete colonization of the developing cerebellum by the Ptc1;p53 genotype leading to the aberrant persistence of a population of self-renewing cells that go on to form metastatic tumors with stem-like characteristics.

We have observed that Ptc1;p53 and Ptc1 represent functionally distinct tumor types and implicated that p53 may be involved in altering the characteristic of the developing tumor. Consistent with our findings, studies of human tumor samples have shown that patients with Gorlin's syndrome, associated with SHH-deregulation, tend to have a tumor histology characterized by extensive nodularity whereas Li-Fraumeni patients carrying mutations germ-line p53 mutations develop medulloblastomas with a classic histology[136]. More generally, histological studies of human medulloblastoma tumors carrying somatic mutations in p53 only very rarely have desmoplastic histology: even the SHH-subtype tumors exhibit classic tumor histology in the context of p53 mutations[137].

The biological mechanisms by which loss of p53 may alter tumor kinetics and properties has been largely attributed to p53-loss triggering extensive and maladaptive DNA rearrangements[138]. By studying the influence of p53 mutation within a developmental context, our work has suggested that p53 loss additionally allows the formation of an aberrant self-renewing population of tumor-initiating cells. Work by the Wechsler-Reya group have utilized a conditional Ptc1-deletion model to demonstrate that a) expansion of a granule cell progenitor is necessary for tumor-formation and b) Ptc1 LOH must occur prior to postnatal day 10 in order to allow tumorigenesis. Our observation that that the Ptc1;p53 but not the Ptc1 genotype results in aberrantly self-renewing neurospheres is consistent with not only an expansion of the population of potential tumor-initiating cells but also a widening of the susceptible time-window in which Ptc1 LOH must occur in order to facilitate tumorigenesis in a manner dependent on loss of p53.

Although p53 has been shown to be involved in adult neural and brain tumor stem cell self-renewal[56,57], the mechanism by which this may occur has been unclear. Indeed, while direct p53 mutations are only rarely found in human medulloblastoma[35], alterations in p53 modulation within these tumors is becoming increasingly appreciated[39,40,57]. Using Sox2-GFP fractionation within the context of the wild-type developing cerebellum we have been able to appreciate gene expressions changes consistent with Id2 modulation of p53 activity representing a potential axis by which p53 could be involved in modulation of stem-cell progenitor pool size (Figure 2-18). It is notable that the Northcott study characterizing differences between early and late-occurring SHH-type medulloblastomas identified Id2 as one of the most differentially expressed genes found in the early-onset tumors[135], consistent with Ptc1 and Ptc1;p53 tumors functionally distinct and suggesting that alterations in cerebellar stem cell differentiation mediated by p53 may play a significant role in determining the properties of these more aggressive and disseminated tumors.

The role of cerebellar stem cells in medulloblastoma

When I first began work in the Jackson-Grusby lab, the idea of a cancer stem cell existing within medulloblastoma was still an emerging concept[58]. At the time, the mechanisms and gene pathways responsible for driving their formation and maintenance were just beginning to be elucidated. The granular cell progenitor was already a well-characterized and accepted cell of origin for Shh-type medulloblastoma formation but was not thought to be at the root of the other types of medulloblastoma[66,90](Figure 4-2). While it is now known that a self-renewing, multipotential stem cell exists in the normal as well as malignant cerebellum[58] and that isolated cerebellar stem cells can indeed be induced to form tumors[68,70], neither the developmental origin of this cell nor the endogenous mechanism of transformation has yet been carefully defined.

The observation of expression of cerebellar stem as well as granular progenitor markers in the clonal Ptc1;p53 BTSC lines suggests either transformation-associated blurring of the distinction between CbSC and GCP or the existence of a common progenitor cell that may possess both Shh-dependent and independent characteristics. Indeed, a recent study has reported that transduction of mutant *MYCN* into embryonic and postnatal cerebellar stem cells led to the development of Shh-dependent and independent tumors, respectively[139]. The validation of Sox2 as a definitive marker for self-renewing cerebellar stem cells using the *Sox2-GFP* reporter animal[98,99] provides a particularly tractable model in which to further characterize the cerebellar stem cells within the context of both normal and malignant development. While immunohistochemical staining for Sox2 in both the adult mouse[68] and human cerebella[101] has marked isolated cells within the cerebellar white matter as well as the Bergmann glia in the Purkinje cell layer, the developmental potency of Sox2-expressing cerebellar cells has yet to be characterized. I have been working to address this question through an ongoing collaboration with the Hochedlinger lab to perform cerebellar lineage-tracing experiments utilizing their *Sox2-CreER*;*ROSA26-Isl-EYFP* mice[98]. By initiating tamoxifen-induced recombination in the early postnatal cerebellum, we are hoping to directly assay the developmental potency of the early Sox2-positive cells within the endogenous wild-type context.

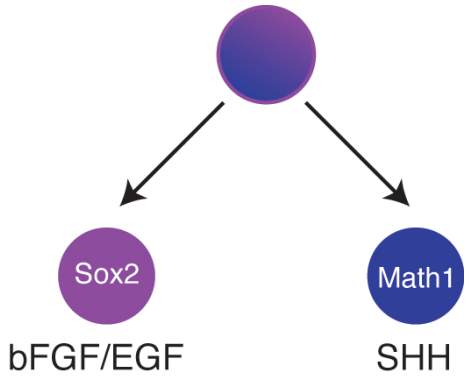

Figure 4-2 | Sox2+ cerebellar stem cells as an alternative medulloblastoma cells of origin
Schematic illustrating the two stem and progenitor cell populations known to be present within the developing cerebellum: bFGF/EGF-dependent Sox2-positive cerebellar stem cells and the SHH-dependent Math1-positive granular cell progenitors.

In order to extend our observations to the mouse tumor models, I have obtained the *Sox2-GFP* and *Sox2-CreER* targeting constructs from the Hochedlinger Lab and modified them for use within our mutant embryonic stem cell lines. Having successfully generated targeted *Ptc1;p53;Sox2-GFP* ES lines, we are currently in the process of producing chimeric medulloblastoma reporter mice. With these animals, we will be able to determine the anatomic origins of the Sox2-positive cells in the developing pre-malignant as well as tumor-bearing cerebellum. Comparisons with the wild-type reporter animals will allow us to identify the origin of these aberrant self-renewing cells. Consistent with our observed lack of self-renewal in the wild-type mature cerebellum, preliminary flow cytometric studies I performed examining a cohort of wild-type reporter animals show a complete lack of identifiable GFP-positive cells in the 3-week cerebellum (data not shown). By contrast, 3-week *Ptc1;p53;Sox2-GFP* chimeric animals show the continued persistence of a distinct population of GFP-positive cells that constitute approximately 10% of the total cerebellum (data not shown). Continued molecular characterization of these cells for gene expression as well as assaying for Ptc1-LOH will be

revealing to see if we are able to capture the earliest cells with tumor-initiating capabilities well prior to overt tumorigenesis.

Furthermore, given that we have already observed gene expression differences following *in vitro* culture, I expect that the use of the *Sox2-GFP* reporter system will additionally facilitate the prospective marking and isolation of the self-renewing, tumor-initiating cells directly from the primary tissue and allow us to more closely examine the *in vivo* state of these cells through gene expression analyses. In this regard, given that I observed significantly increased numbers of CD15- and CD133-marked cells in the Sox2-positive fraction, we will investigate whether sorting for CD15/CD133/Sox2 triple-positive cells will further enrich for the bio-active population of cells. I have previously seen that direct injection of $5x10^4$ unfractionated Ptc1;p53 primary tumor cells gives rise to cerebellar tumors over a period between 5 and 10 weeks and am interested to see how enrichment for these self-renewing cells may increase tumorigenic potential.

While there are a number of clinical studies for medulloblastoma focused on using SHH-pathway antagonists as single-agent treatment protocols, I hope that the work presented here taken in conjunction with the increasing appreciation throughout the field of both inter- and intra-tumor heterogeneity will cause us to design and develop multi-pronged therapeutic approaches that will simultaneously target multiple aspects of tumor cell biology. Specifically, the observation that while treatment with SHH-antagonists in both mice[43,45] and human[44] medulloblastoma may delay tumor progression, it is not, in most cases, curative. The appreciation and further characterization of a Sox2-positive bFGF/EGF-dependent cell of origin

for medulloblastoma through the use of GFP-reporter system ,will allow the elucidation of novel pathways that will hopefully contribute to the development of new treatment paradigms for Shh-subtype tumors.

Finally, our work in characterizing the epigenetic state of the Ptc1;p53 BTSC has identified a potentially promising and novel mechanism by which BTSC are able to resist differentiation. I have generated four new lentiviral hairpins targeted against the Kcnq1ot1 RNA to further substantiate the role of Kcnq1ot1-mediated regulation of Cdkn1c in maintenance of BTSC self-renewal. The parallel use of the *Sox2-GFP* system here will allow us to ask if bi-allelic expression of the *Kcnq1ot1* locus can be directly observed in the self-renewing cells of the developing and tumor-bearing cerebellum. Lastly, the ability to isolate highly pure populations of cerebellar stem cells from both wild-type and mutant animals opens the door for high-throughput screens that will allow us to compare the genetic and epigenetic states of these cells and gain insight into the genes and pathways involved in modulating their existence.

Developmental field cancerization and human malignancies

In its initial description by Slaughter[92], the phenomena of field cancerization was observed in epithelial malignancies wherein predisposing mutations, often associated with p53, conferred a growth advantage to those cells allowing the out-competition of wild-type cells in the field and facilitating the formation of a 'patch' or 'field' of pre-malignant cells that could subsequently give rise to malignancy. Within this framework, field cancerization preceding malignant transformation has been used to describe tumors associated with, but not limited to, Barrett's esophagus secondary to acid-reflux[140], multifocal and recurrent squamous cell cancers of the

upper aerodigestive tract in the context of tobacco-use and other carcinogens[141], hepatocellular carcinoma occurring secondary to hepatitis-infection[142], ulcerative colitis associated neoplasia[143], and gastrointestinal cancers arising in the context of *Helicobacter pylori* infection[144]. As thus conceptualized, properties considered for the occurrence of field cancerization may consist of a tissue system that undergoes continuous cellular turn-over as well as an ongoing source of external insult or cellular injury that may be facilitating the accumulation of stochastic mutation or cellular turn-over, respectively.

The choice of using a somatic mosaic model for studying medulloblastoma formation allowed us to make a serendipitous observation that would not have been otherwise possible: Ptc1;p53 cells are able to non-randomly and preferentially seed the developing cerebellum such that nearly 100% of the chimeric animals developed medulloblastomas. In observing the dramatic and specific pre-malignant seeding of cerebellar field prior to tumorigenesis, we are seeing a tissue-specific competitive growth advantage for the Ptc1;p53 mutant cells which we have been able to attribute to Ptc1 haploinsufficiency. Thus, the first hit of the *Ptc1* gene has, contrary to Knudson's two-hit hypothesis[106], not a neutral, but a very significant influence on future disease susceptibility. More broadly, these observations suggest that germ-line inheritance of tumor suppressor genes does not have to occur in order to pre-dispose an individual to future malignancy. More generally, we have been able to re-frame field cancerization within a developmental context characterized by wide-spread and rapid cellular proliferation, differentiation, and turnover, and illustrate how preferential dependence on a given morphogen tumor suppressor pathway in particular organ systems may confer profound influence to

corresponding *de novo* tumor suppressor gene mutations occurring during early embryonic and organismal development.

These conclusions naturally bring to mind the observations that mutations in some widely expressed tumor suppressor genes are associated with characteristic tumor spectra: *Rb* mutations with retinoblastoma, *BRCA* with breast and ovarian cancers, *NF1* with neurofibromatosis-1, *APC* with colon cancer, etc[145]. One would expect that analogous experiments performed with mouse ES cells heterozygous for tumor suppressor genes other than *Ptc1* would exhibit preferential and distinct tissue colonization reflective of the expected tumor spectra for a given gene mutation. For instance, the developmental expression of the *BRCA* genes is consistent with their playing a critical role in the differentiation of breast epithelia[146,147] and is further substantiated by observations that BRCA1 depletion by RNAi leads to impaired differentiation and enhanced proliferation of breast epithelial cells[148].

If developmental field cancerization were more broadly applicable, it could influence the framework in which we conceptualize tumorigenesis. Up until now, tumor evolution has been primarily modeled by the accumulation of mutations within small cell populations through progressive Darwinian selection[149]. However, most models are not able to reconcile differences between the number of mutations thought to be needed for tumor progression and the spontaneous mutation rate of normal cells, and thus posit that acquisition of a 'mutator phenotype' or genetic instability is necessary for multistage tumorigenesis[150]. Computation work modeling the dynamics of tumor suppressor gene inactivation has suggested that with extremely large starting populations, the waiting time prior to complete tumor suppressor gene activation

becomes exceedingly short[151]. Developmental field cancerization could allow for the generation of just such a large population from an otherwise 'neutral' tumor suppressor gene mutation, allowing for unhindered expansion of the pre-malignant field. In this context, *Ptc1*-haploinsufficiency mediated colonization of the cerebellum in medulloblastoma formation involves potentially 25-30 million granular neurons in mice[152] ; the susceptible cerebellar field in humans is thought to include approximately 50 billion granular neurons[153].

Within humans, evidence for significant somatic mosaicism has already been reported in retinoblastoma[154] as well as neurofibromatosis patients[155].

Given our ever-increasing ability to differentiate between normal and tumor tissue on a genetic level, it is my belief that the observation of developmental field cancerization in humans is imminently possible.

Chapter 5 References

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